EFFECTIVE IMMUNOTHERAPY OF ADVANCED CANCER IN MICE

Dissertation

zur
Erlangung der naturwissenschaftlichen Doktorwürde
(Dr. sc. nat.)

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2. Summary

The immune system plays a significant role during tumorigenesis with evidence for a promoting as well as a suppressing role. Indeed, the amount and location of tumor-infiltrating Th1 and cytotoxic T cells is a strong positive prognostic factor in multiple types of human cancers. However, advanced tumors have intrinsic capacities to evade immune attack (immunoediting) as well as extrinsic mechanisms including a suppressive microenvironment, which also dampens tumor-specific immunity.

The main aim of immunotherapy is to improve protective effector function of tumor-specific T cells, which relies on 3 signals: Stimulation of the TCR receptor (signal 1), costimulation (signal 2) and supportive cytokines (signal 3). In the context of cancer, naïve T cells are insufficiently primed and become progressively dysfunctional or tolerant most probably due to the low quality of these 3 signals. Although the requirements for T cell activation are well characterized, how to prevent tolerance in the context of cancer remains unclear.

Boosting antitumor responses by blocking PD-1 or CTLA-4 results in durable clinical responses only in a limited proportion of cancer patients suggesting that other pathways must be targeted to improve clinical efficacy. We therefore aimed to develop an approach to prime tumor-specific $CD8^+$ T cells, prevent induction of tolerance and achieve control of large, established tumors. We used mice that develop autochthonous prostate cancer (TRAMP mice) as well as mice carrying advanced melanoma (B16) and combined different immune interventions with adoptive transfer of tumor-specific TCR transgenic $CD8^+$ T cells.

We found that the combination of agonistic anti-CD40 + IL-2/anti-IL-2 complexes + IL-12Fc was a distinctively effective treatment with respect to priming protective, tumor-specific immunity and eradicating tumors at advanced disease stage when given together with adoptively transferred tumor-specific T cells. We propose that improving signals 2 (costimulation) and 3 (cytokines) together with fresh tumor-specific rather than dysfunctional pre-existing memory T cells represents a potent therapy for advanced cancer.

The second part of this thesis describes the generation of a novel inducible mouse model of cancer.

3. Zusammenfassung

Das Immunsystem spielt eine bedeutende Rolle bei der Tumorgenese und es gibt Belege dafür, dass das Immunsystem sowohl eine fördernde, als auch eine unterdrückende Rolle bei der Tumorentstehung spielt. In der Tat sind Anzahl und Lokalisation der tumor-infiltrierenden Th1 und zytotoxischen T-Zellen ein bedeutender positiver prognostischer Faktor bei verschiedenen Krebserkrankungen des Menschen. Fortgeschrittene Tumore haben intrinsische Kapazitäten ("immunoediting") und extrinsische Mechanismen um sich gegen Angriffe des Immunsystems zu wehren; unter anderem führt die suppressive Tumormikroumgebung dazu, dass die tumor-spezifische Immunität gehemmt wird.

Das Ziel der Immuntherapie ist es die Effektorfunktion von tumor-spezifischen T-Zellen zu verbessern, welche auf 3 Signale setzt: Stimulation des T-Zell-Rezeptors (Signal 1), Kostimulation (Signal 2) und Freisetzung von unterstützenden Zytokinen (Signal 3). Im Zusammenhang mit Krebs werden naive T-Zellen unzureichend "geprimed" und aufgrund der geringen Qualität der 3 Signale zunehmend dysfunktional oder tolerogen. Obwohl die Voraussetzungen der T-Zellaktivierung gut charakterisiert sind, ist es weiterhin unklar wie die Toleranzentstehung im Zusammenhang mit Krebs verhindert werden kann.

Eine Verbesserung der Antitumorimmunität durch Blockierung von PD-1 oder CTLA-4 ergibt nur in einem begrenzten Anteil an Krebspatienten einen dauerhaften klinischen Erfolg, was darauf hindeutet, dass andere Wege gewählt werden müssen um die klinische Wirksamkeit gezielt zu verbessern. Daher bestand das Ziel darin, einen Therapieansatz zu entwickeln, der die tumor-spezifischen $CD8^+$ T-Zellen primed, Toleranzentstehung verhindert und das Wachstum von großen, etablierten Tumoren kontrolliert. Hierfür verwendeten wir ein Mausmodell für autochthonen Prostatakrebs (TRAMP Mäuse), sowie Mäuse mit fortgeschrittenem Melanom (B16) und kombinierten verschiedene Immuninterventionen mit einem adoptiven Transfer von tumor-spezifischen T-Zell-Rezeptor transgenen $CD8^+$ T-Zellen.

Wir fanden heraus, dass die Kombination von agonistischem anti-CD40 + IL-2 / anti-IL-2-Komplexe + IL-12Fc einen bedeutenden Behandlungeffekt erzielte. Die Kombination führte zu einer schützenden, tumor-spezifischen Immunität und zu einer Ausrottung von Tumoren im fortgeschrittenem Krankheitsstadium, wenn sie zusammen mit adoptiv transferierten tumor-spezifischen T-Zellen gegeben wurde. Wir schlagen vor, dass die Verbesserung der Signale 2 (Kostimulation) und 3 (Zytokine) zusammen mit tumor-spezifischen, und nicht mit dysfunktionalen vorbestehenden Gedächtnis-T-Zellen, eine wirksame Therapie bei Krebs im fortgeschrittenen Stadium darstellt. Der zweite Teil der Arbeit beschreibt die Erzeugung eines neuartigen induzierbaren Tumor-Mausmodell.

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5. Introduction

5.1. A brief history of cancer

Since the oldest description of cancer in an Egyptian papyrus dating back to the seventeenth century B.C., our understanding of this disease has tremendously improved. Although the papyrus is the first documentation of the disease, the first usage of the word cancer goes back to the father of medicine, a Greek physician from the fourth century B.C. named Hippocrates. Almost four millenniums later, the treatment of cancer remains, in many cases, regrettably similar to what is written in the Edwin Smith Papyrus, which denotes it as an incurable ailment (*1*).

Nowadays, cancer figures in the leading causes of morbidity and mortality worldwide. For 2012, it was estimated that 14 million new cancer cases were diagnosed globally and this figure is projected to grow by 70% in the next 2 decades (*2*). Advances in modern medicine significantly improved the expected survival of cancer patients, but many cancers remain incurable although a large proportion could be prevented. Indeed, a third of cancer-related deaths could be avoided by behavioral and dietary changes, with the main cancer risk factors consisting of a high body mass index, an unhealthy diet, a lack of physical activity and consumption of tobacco and alcohol.

Even if cancer has been known for millenniums, our current understanding of it consists largely in the research discoveries made since Richard M. Nixon declared war on cancer with the National Cancer Act of 1971. Since this historical date, colossal investments over the globe accelerated the pace of research in the quest for developing better treatments.

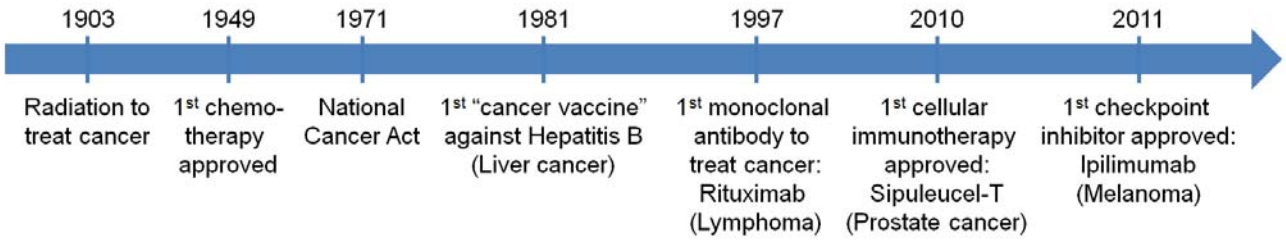

Figure 1. Some major milestones of the fight against cancer.

5.2. Our current view of cancer

5.2.1. Hallmarks of cancer

The transformation of a normal cell into a cancer cell consists of a multistep process starting with genetic aberrations that lead to gain- or loss-of-function mutations of crucial regulating genes (*3*, *4*). The number and location of these mutations vary greatly among cancer types, but their common feature is that they allow the cell to progressively evolve towards a neoplastic state. The process is analogous to Darwinian selection and leads to the acquisition of several hallmark capabilities increasing the cell's capacity to sustain proliferation, resist cell death, induce angiogenesis and activate invasion and metastasis mechanisms. These hallmarks were recently updated to acknowledge the important role of tumor-promoting inflammation, enhanced genomic instability, deregulated metabolism and avoidance of immune destruction in tumorigenesis. Collectively, these acquired features summarized in Figure 2 (*4*) dictate cancerous growth. This simple schematic of complex mechanisms provides a recent overview in which all pathways are under intense investigation to generate novel targeted therapies.

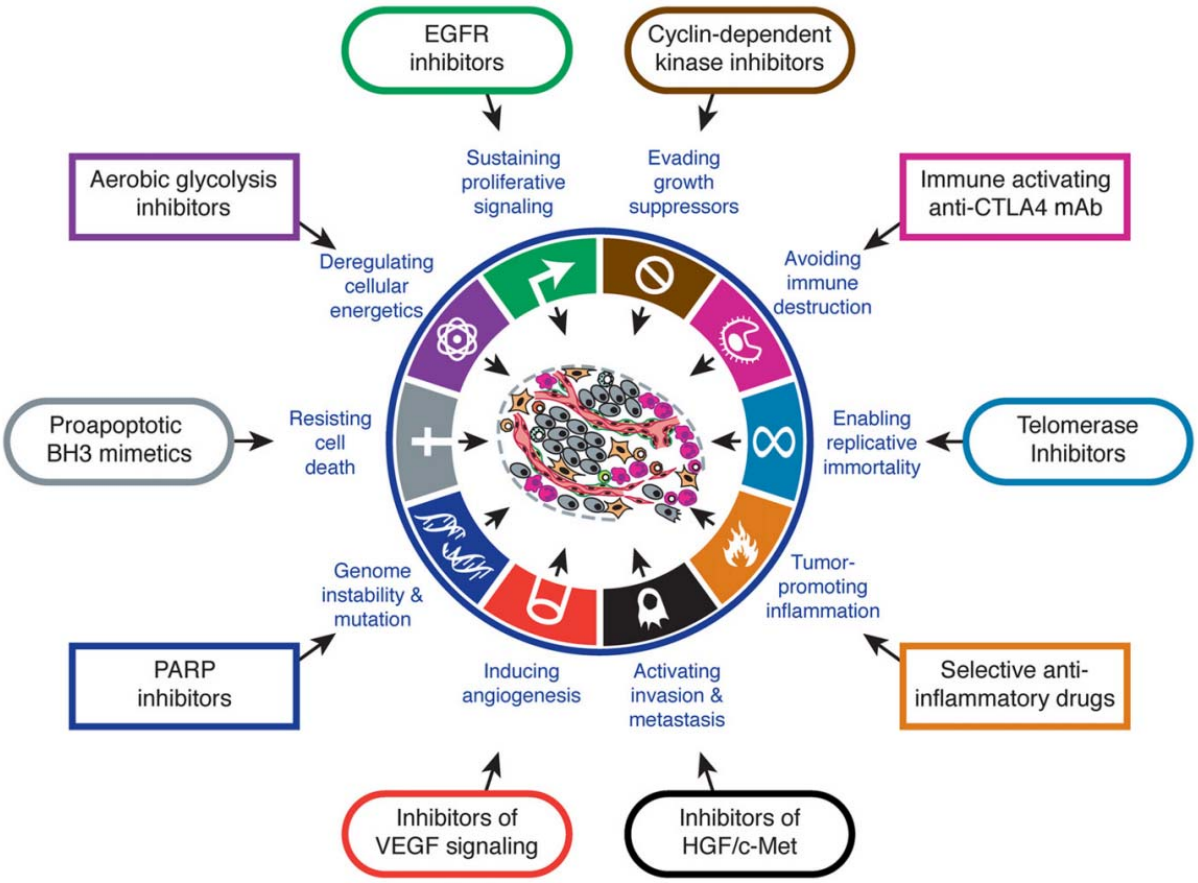

Figure 2. Hallmarks of cancer (4).

5.2.2. Cancer immunosurveillance

The growing comprehension of each of these processes has reshaped the way modern medicine tackles cancer and has triggered the expansion of promising areas of research. Although not new, the field of tumor immunology is one of them. In 1909, Paul Ehrlich suggested for the first time that the immune system was suppressing cancer development (*5*). Five decades later, Burnet and Thomas reformulated this theory as the "cancer immunosurveillance" hypothesis speculating that lymphocytes are sentinels of the host that continuously eliminate arising transformed cells (*6-8*). This concept suffered early on from the lack of strong experimental evidence and was buried after the report that immunodeficient nude mice (*9*) don't show increased frequency of spontaneous or chemically induced tumorigenesis (*10*). Multiple other studies (*11-13*) were conducted on this topic confirming Stutman's results which provided highly convincing data leading to the abandonment of the immunosurveillance theory in the 1970's. However, it is now clear that the limited knowledge available at that time about the immunologic impairments of nude mice misguided the scientific community on the immune defense to cancer.

5.2.3. Cancer immunoediting

Recently, tumor evasion of immune surveillance arose again as a major topic in cancer research and comes from convincing new data in mice and humans that proved the importance of the immune system in controlling cancer. The questions are not anymore about the existence of the process, but rather how to translate it to improve cancer therapies. To progress in this direction, it is crucial to decipher the complex interplay between tumors and the immune system. On the one hand, recent results indicated that both the innate and adaptive arms of the immune system contribute to tumor cell control (*14-17*). On the other hand, tumor immunity has been shown to have tumor-promoting roles through the selection of tumor variants that escaped immune recognition and elimination (*15, 16, 18*). This double-edge sword consisting of tumor destruction and promotion of tumor growth has been summarized into the cancer immunoediting process (Figure 3).

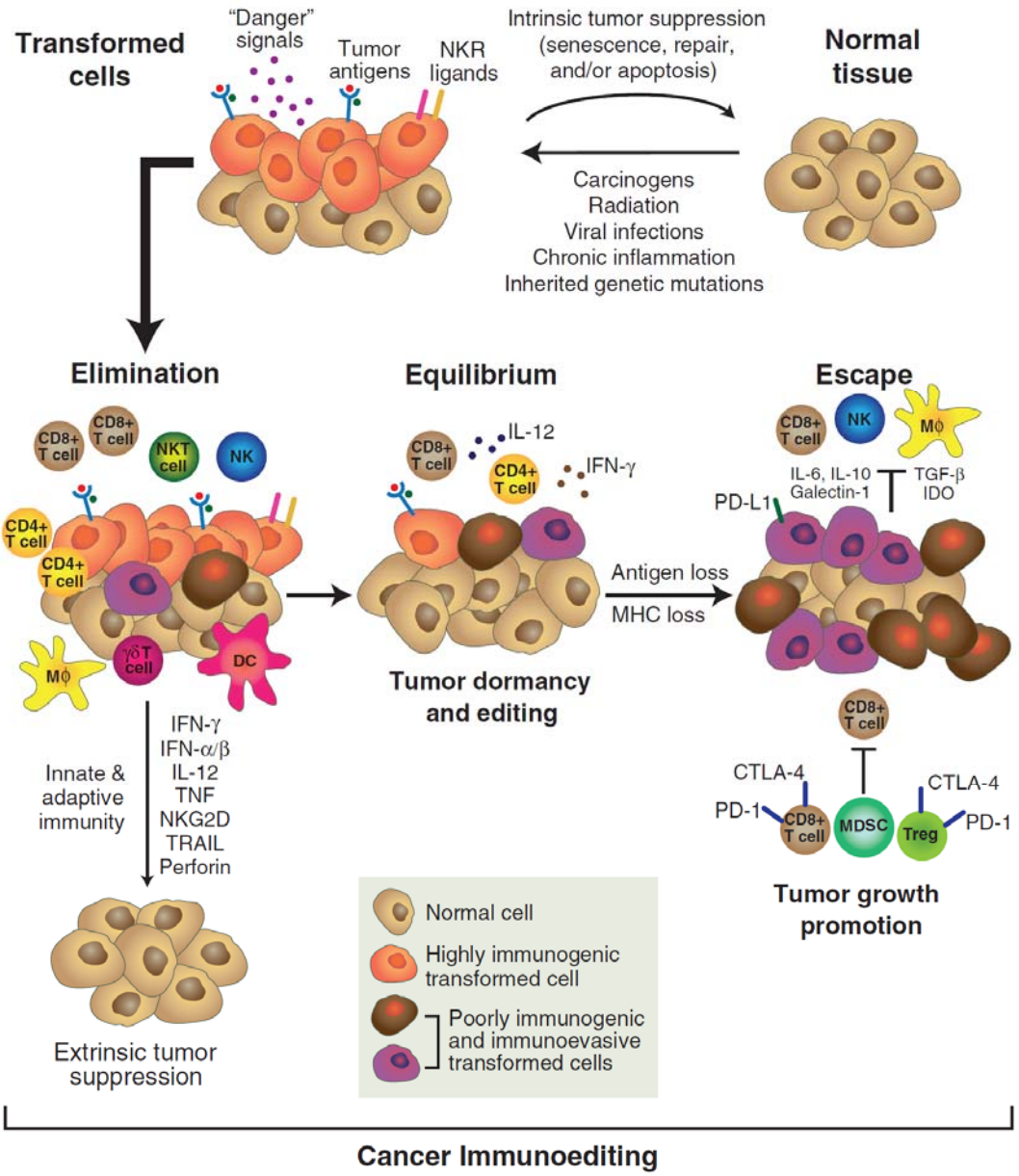

Figure 3. The three Es of cancer immunoediting (16).

As illustrated in Figure 3, the phases of elimination, equilibrium and escape are thought to occur in a sequential manner following a Darwinian model of immunological pressure on tumor cells. However, the concept doesn't exclude that some cells may directly enter either into equilibrium or escape phase.

5.2.3.1. Elimination

The elimination represents the phase in which innate and adaptive immunity cooperate to sense and destroy arising transformed cells. It is not fully understood how the immune system detects such cells, but multiple propositions have been made. Multiple danger signals were described including type I interferons (*19*), damage-associated molecular pattern molecules

(DAMPs) released from dying cells and damaged tissues (*20*) as well as NKG2D stress ligands (*21*). All these mechanisms eventually converge towards the release of pro-inflammatory and immunomodulatory cytokines that supports the establishment of a tumor-specific adaptive immune response. Although it has been reported in some models that innate immunity was sufficient to protect against cancer growth (*21-23*), in most experimental systems, an effective cancer immunosurveillance requires the additional involvement of adaptive immunity. The strongest evidence for the elimination phase in humans consists of the spontaneous regression of melanoma lesions accompanied by the clonal expansion of tumor-specific T cells (*24, 25*). However, cases of spontaneous tumor regression remain rare events.

5.2.3.2. Equilibrium

The equilibrium phase consists of a balance between tumor control by the immune system and tumor growth yielding a net result neither in favor of elimination nor progression. Immunoediting takes place during this latency phase with the immune system eliminating tumor cells that are more immunogenic and therefore selects for variants with a higher potential to evade immune control. This process was demonstrated in mice with two different approaches. The first experimental proof came from transplantation of tumors that grew in wild-type or immunodeficient mice with a targeted disruption of the recombination-activating gene-2 (RAG2) and therefore lacks functional B, T and NKT cells. When these tumors were transplanted into wild-type recipients, the tumor growth differed depending on their origin. As illustrated in Figure 4, tumors growing in $RAG2^{-/-}$ mice were considered unedited, rendering them more susceptible to rejection in an immunocompetent host while edited primary tumors that grew in wild-type mice could evade immune control in immunocompetent recipients (*18*).

A second strong evidence of the equilibrium phase was demonstrated using low dose of the chemical carcinogen 3'-methylcholanthrene (MCA) to induce carcinogenesis in mice. As shown in Figure 5, the equilibrium represented by the absence of detectable tumors in MCA-treated animals can be disrupted by antibodies depleting T cells and neutralizing IFN-γ showing the importance of constant control by adaptive immunity over long periods of time. This is observed in humans for example by the development of donor-derived cancers in organ transplant recipients (*26*). Further clinical observations supporting the existence of the equilibrium phase in humans are represented by the long period between successful treatment, detection of minimal residual disease and relapse (*27, 28*).

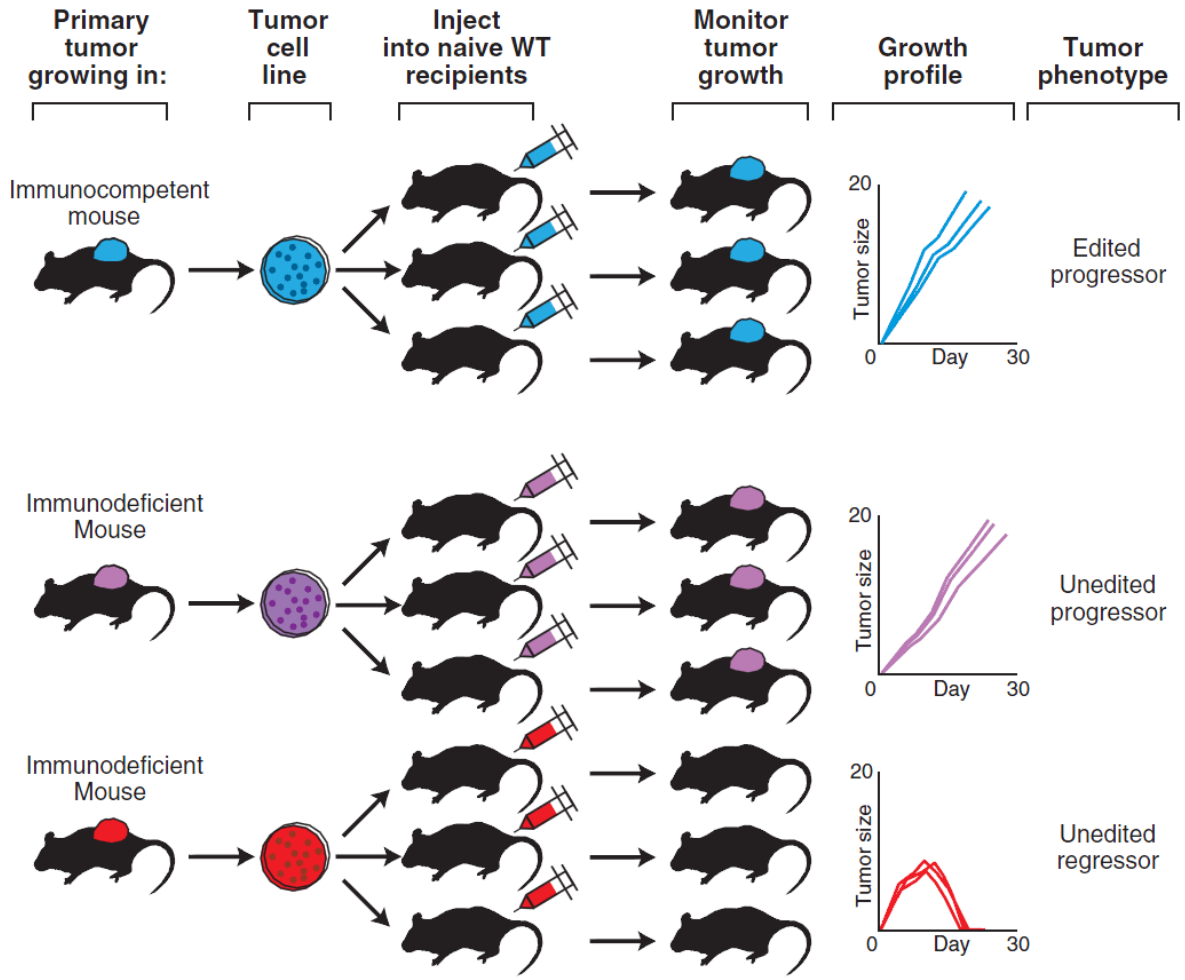

Figure 4. Immunoediting of cancer cells (16).

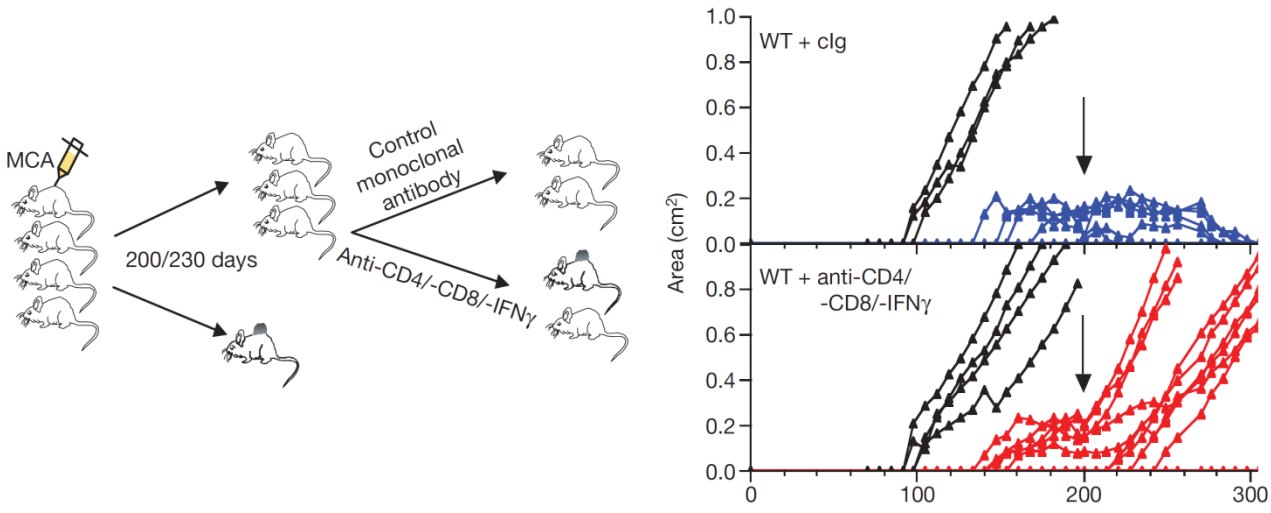

Figure 5. Antibody-induced equilibrium disruption in mice (29).

5.2.3.3. Escape

The escape of tumor cells from immune control occurs when the equilibrium can't be maintained and tumor-specific immunity becomes insufficient to limit cancer progression. Multiple intrinsic and extrinsic mechanisms have been identified that promote tumor's escape from immune attack. Inherent capacities acquired by tumor cells, for example reduced immune recognition through the loss of tumor antigens (*30*), impaired antigen presentation, improved resistance to cell death and expressions of surface molecules or soluble factors creating an immunosuppressive microenvironment can all contribute to tumor cell survival (*31*). Additionally, extrinsic mechanisms favoring tumor survival and growth include numerous cell types recruited to tumors (MDSCs, Treg, macrophages) and the factors they produce locally that impact on tumor-specific immunity. The number of molecules contributing to the escape phase continuously increases and these pathways represents attractive targets for cancer therapy (*31*).

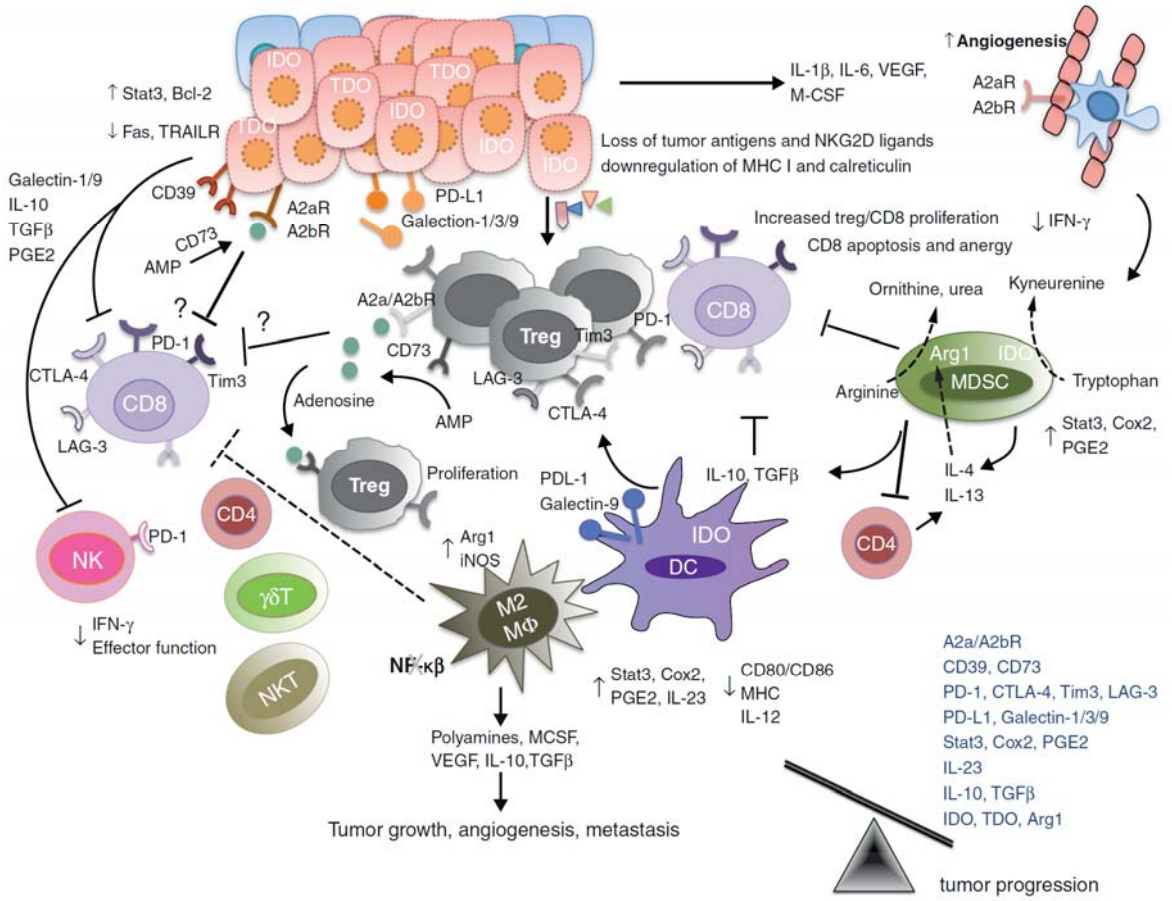

Figure 6. Mechanisms contributing to the escape of tumor cells (31).

Although each step of cancer immunoediting is supported by strong evidence, it remains unclear how frequent and how rapidly these phases occur in humans. Recent data acquired using multiregion whole-genome sequencing of operable non-small cell lung cancers suggested a prolonged tumor latency period of at least 20 years before clinical detection in former smokers (*32*).

Certainly, the length of the equilibrium phase characterized by tumor dormancy and editing may vary significantly between patients and also among cancer types, but this strongly supports the rationale to continuously improve diagnostic methods to allow earlier detection. There is also clinical information supporting the existence of proliferative dormancy at premetastatic sites (*27, 33, 34*).

Therefore, the equilibrium phase which comprises an ongoing immune response would be, in theory, the best window to treat patients. Unfortunately, most patients are diagnosed in the escape phase, when the equilibrium is compromised and the tumor progresses in a microenvironment rich in immunosuppressive signals that may dampen the efficacy of current therapies. Ongoing efforts in the field aim at restoring this equilibrium phase through immunomodulation and in the best case scenario, to eradicate cancer.

5.3. Harnessing the immune system against cancer

5.3.1. Immune infiltrate: prognostic significance

In several human cancers, it has been demonstrated that the type, density and localization of immune cells infiltrating the primary tumor constitute strong prognostic factors of overall survival relevant at any stages of the clinical disease (*35, 36*).

More specifically, high infiltration by memory T cells of the Th1 and cytotoxic types provides patients with better survival chances as shown in Figure 7 (*37*). Concerning other immune cell subsets, no consensus has been established, but the majority of publications consider Th2, Th17 and Treg cells to be detrimental to the patients. With the complexity of the tumor micro-environment, it remains unclear if T lymphocytes priming is limited to secondary lymphoid organs before homing at the tumor site, but it was recently suggested that tertiary lymphoid structures (TLS) may support priming of adaptive immunity (*38-40*). The abundance of these structures composed of mature dendritic cells adjacent to T cell clusters and B cell proliferating germinal center correlates with better survival in multiple cancer types and with increased CD8 T cell infiltration, which indirectly supports this hypothesis (*40-43*). In accordance with this theory, it was recently reported that the density of TLS-associated

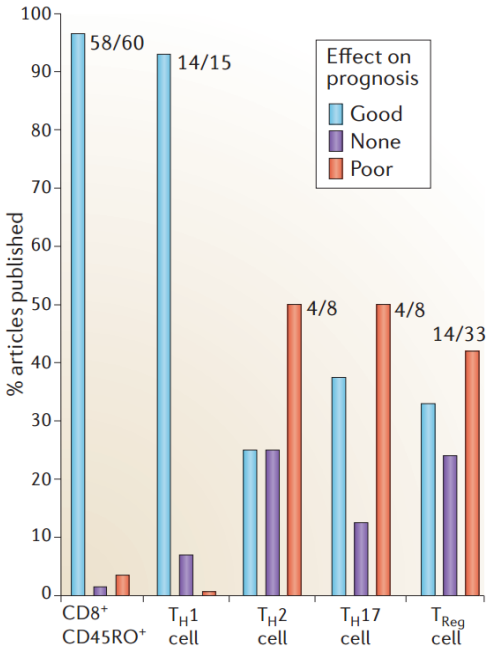

Figure 7. Association of immune infiltrate with prognosis in various types of cancer (37).

mature DC correlates with a gene signature related to T cell activation of Th1 and cytotoxic phenotype in human primary lung tumors (*39*). It will be interesting to know if the TLS adjacent to tumors represent an essential part of the immune contexture to mount or support an effective tumor-specific immune response.

Recent studies provided evidence that responses to checkpoint blockade immunotherapy are better when pre-existing tumor-specific immunity is present in cancer patients (*44-47*). Interestingly, these results showed that adaptive immune responses observed in mice and patients treated with checkpoint blockade targeted tumor-specific mutant antigens. In line with these findings, the mutational load in cancer patients was shown to be associated with the degree of clinical benefit to such therapy (*47*). The discovery of a neoantigen landscape that is specifically present in strong responders to anti-CTLA-4 therapy provides a rationale for examining exomes of patients for whom immunotherapy is being considered. These tumor-specific mutant antigens also offer an opportunity to develop personalized cancer-specific vaccines.

5.3.2. The 3 signals crucial for T cell priming

Multiple events are required for optimal priming of T cells. First, the T cell must interact with its cognate peptide on the antigen-presenting cell (APC) through TCR-mediated binding of the MHC complex presenting the peptide epitope. This event occurs through the establishment of a supra molecular activation cluster (SMAC) also known as the immunological synapse between the T cell and the antigen-presenting cell (*48-50*).

During this cell-cell contact, the second event consists of crucial costimulatory signals to dictate differentiation and proliferation of the T cell. At this step, the maturation status of the APC is a major factor regulating the balance between costimulatory and coinhibitory signaling (*51*). Figure 8 illustrates the numerous molecules influencing this balance. These early signaling events through the TCR and costimulatory molecules trigger a cascade of intracellular events in T cells including tyrosine kinase activation, inositol phospholipid hydrolysis and a surge in intracellular Ca^{2+} (*52*). Downstream, the CBM complex composed of Card11, Malt1 and Bcl10 amplifies the signal through its particular scaffold structure. This complex is a central player in T cell activation and leads to optimal NF-κB activation (*52*). At the same time, multiple other pathways culminate in the translocation to the nucleus of several transcription factors involved in the production of IL-2 and other T cell activation signature genes programming a transcription profile that regulates cytoskeletal rearrangements, cellular metabolism, cell cycle and cell differentiation (*52-54*).

Autocrine and paracrine IL-2 represents one of the most important cytokine supporting T cell survival, proliferation and differentiation (*55*). Nonetheless, multiple other cytokines influence

T cell faith and constitute signal 3, which is mainly provided by IL-12 or type I interferons (*56-58*). Altogether, antigen affinity, costimulation and cytokine inputs dictate T cells expansion, differentiation into effector cells and establishment a memory population (*59*).

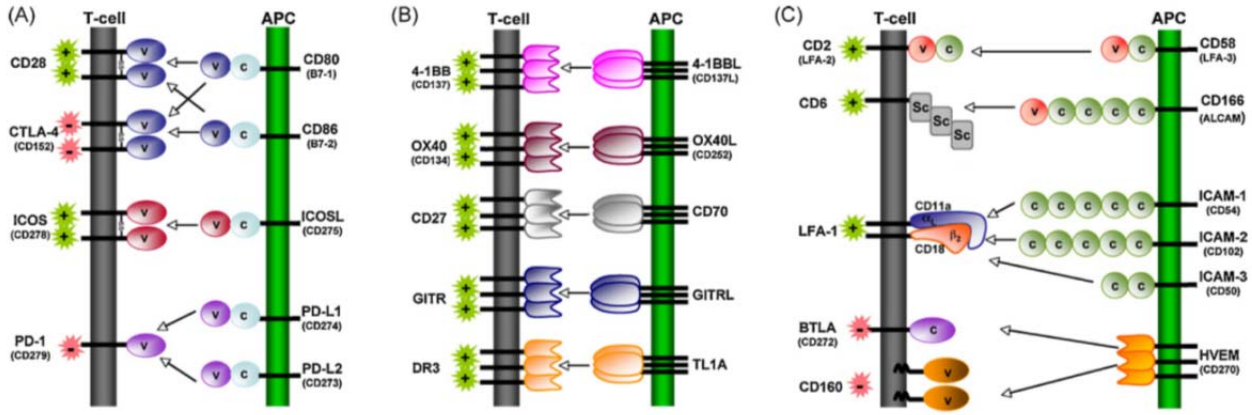

Figure 8. Costimulatory and coinhibitory molecules on T cells and their target (60).

5.3.3. Enhancing tumor-specific immunity through therapy

As mentioned previously, both the innate and adaptive arms of the immune system can play a beneficial role in tumor control. Therefore, an optimal therapy should impact on both compartments to yield protective and long-lasting tumor-specific immunity.

It is now clear that conventional therapies like radio- and chemotherapy previously thought to exert their efficacy mainly through direct toxicity to tumor cells also support tumor-specific immunity, which contributes to clinical responses (*61-67*). This suggests that immune stimulation can synergize with such therapies and multiple studies support this concept in mice (*68-70*), as well as in humans (*71-78*). Currently, this knowledge is being translated into a growing number of clinical trials, although trial results remain scarce so far.

Immunotherapeutic approaches including vaccines (*79*), adoptive T cell transfers (*80*), induction of APC maturation (*81, 82*), checkpoint blockade (*83, 84*) and cytokines (*85, 86*) improve tumor-specific immunity in mice and humans to a certain extent, but objective clinical responses remain limited. Although promising, these results suggest that additional pathways need to be targeted to increase clinical efficacy. All of these approaches ultimately aim by different ways to improve the effector function of T cells which relies on the 3 signals previously mentioned: Stimulation of the TCR receptor (signal 1), costimulation (signal 2) and supportive cytokines (signal 3) (*52*). In the context of cancer, antigen-presenting cells do not adequately deliver signals 2 and 3, which leads to robust, antigen-specific T cell tolerance (*51, 56, 57, 87, 88*). While the requirements for T cell activation are well characterized, the optimal way to prevent T cell tolerance in the context of cancer remains unclear.

5.4. Aims

One of the current challenges in the field of cancer research resides in the difficulty to extrapolate preclinical findings to humans. This issue may never be solved completely, but during the past decade, the field gained awareness and understanding about the critical need for better preclinical cancer models (*89-92*). Spontaneous, autochthonous cancer models develop more slowly than subcutaneously injected cancers and give rise to cancer in the physiological tissue context, both of which mimic human cancer.

This thesis focused on understanding and counteracting tumor-specific $CD8^+$ T cell unresponsiveness in the context of cancer and two projects were pursued in parallel. The first part was dedicated to developing an immunotherapeutic intervention that results in priming of tumor-specific $CD8^+$ T cells, prevention of immune deviation and clinical response. This project was performed using a well-characterized autochthonous model of prostate cancer (TRAMP) (*93*). The carcinogenesis in this model is SV40 large T antigen (SV40LT)-dependent, which enabled the use of multiple SV40LT-related tools that were generated or acquired at the beginning of this study. In addition, prostate cancer represents the second leading cause of cancer deaths in men and remains the cancer with the highest incidence according to statistics collected by the American Cancer Society for 2014 (*94*).

In line with the need for better models for cancer in different tissues, the second project of this thesis consisted of generating a new preclinical cancer model (ARTURO) to allow inducible tissue-specific tumorigenesis (Cre^{ERT}/LoxP system) through the expression of SV40LT.

5.5. Transgenic adenocarcinoma of the mouse prostate

The TRAMP mouse (transgenic adenocarcinoma of the mouse prostate) develops autochthonous prostate cancer as a result of prostate-specific expression of SV40LT under the control of the rat probasin promoter (*93*). In this model, the expression of the large T antigen is induced upon sexual maturation of male mice since the promoter is androgen responsive. As illustrated in Figure 9, hemizygous transgenic animals progress through various phases of disease from mild intraepithelial hyperplasia at puberty to large malignant neoplasia within the appropriate microenvironment (*95, 96*). Histological grading in this model has been established and consists of 6 stages described in Table 1 (*95*). The TRAMP model has attracted widespread attention in the last two decades due to its ability to closely mirror the multistep pathogenesis seen in man (*97-99*). Moreover, androgen-driven and developmentally regulated tumors transiently regress following androgen withdrawal, but subsequently relapse, paralleling the eventual occurrence of deadly hormone-independent prostate cancer observed in man (*100, 101*). Therefore, the TRAMP model

represents a clinically relevant murine model of prostate cancer. However, the TRAMP model has disadvantages including the lack of methods to quantify tumor load in live animals and the long timeframe of tumor development. These are obviously major drawbacks, as clinical response is the most relevant parameter for any therapy that aims to improve tumor-specific immunity. The use of ARTURO mice intended to solve this problem, because ARTURO mice allow non-invasive and repeated quantification of tumor load of Cre-recombined tumor cells expressing luciferase (luc) with the SV40LT. Early phases of my work also aimed at establishing novel experimental setups in TRAMP mice that would allow faster assessment of clinical efficacy.

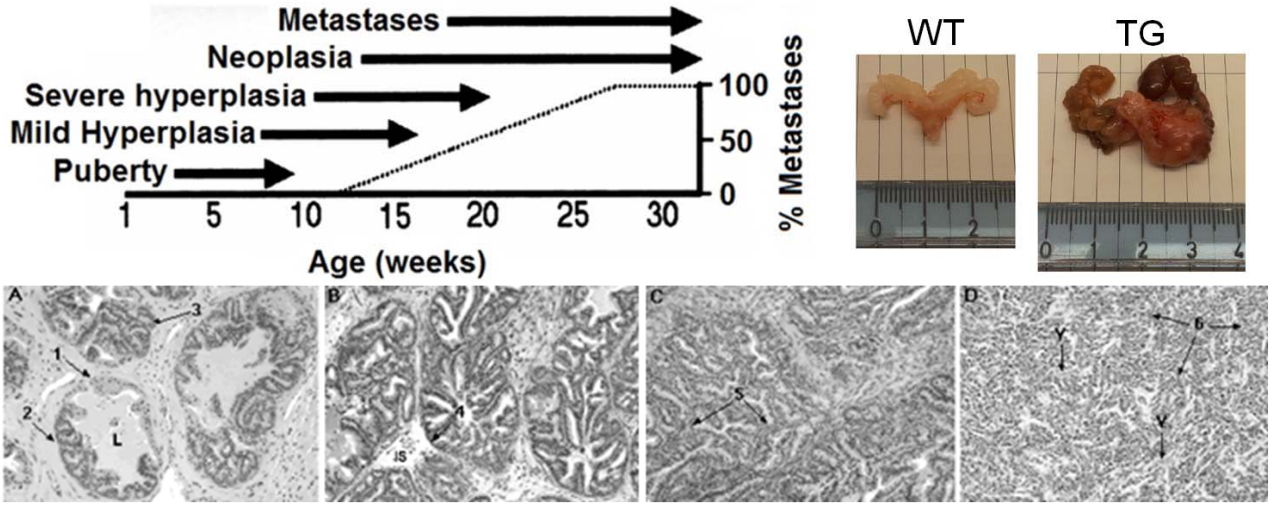

Figure 9. Progressive carcinogenesis in TRAMP mice (95, 96).

Histopathic score	Histopathic classification	Description
1	Normal prostate	In the normal prostate, epithelial cells comprising the glands are uniform in size and orientation, and their nuclei are small and well-defined.
2	Early intraepithelial neoplasia	Early neoplastic changes are evident as an increase in epithelial nuclear-to-cytoplasmic ratio and "tufting up" of the epithelial layer or layers into the glandular lumen. This lesion is analogous to a low grade PIN lesion in humans.
3	Advanced intraepithelial neoplasia	This lesion is analogous to a more-advanced PIN lesion in humans and is associated with extensive infolding of epithelial cell layers into the lumen and an increase in both mitotic and/or apoptotic figures.
4	Well-differentiated prostate adenocarcinoma	This grade of cancer is represented by early invasion/penetration of the glandular basement membrane by tumor cells that extend into the stromal compartment
5	Moderately differentiated prostate adenocarcinoma	Moderately-differentiated cancer is represented by tumor formation of primitive glands lacking an obvious lumen. Tumor cells comprising these glands have lost their tall secretory appearance.
6	Poorly differentiated adenocarcinoma	This most severe grade of prostate cancer is represented by tumors composed of sheets and cords of highly pleiomorphic anaplastic tumor cells.

Table 1. Histopathologic grading of TRAMP tumors, adapted from (95).

6. Results

6.1. Effective immunotherapy of advanced cancer in mice

Submitted manuscript.

Title: Effective immunotherapy of advanced cancer in mice

Authors: Ali Bransi, Oscar Camilo Salgado, Michal Beffinger, Karim Milo, Hideo Yagita, Burkhard Becher, Alexander Knuth, Maries van den Broek

Contribution: AB and MvdB designed experiments. AK and MvdB supervised the study. AB performed most experiments. OCS, KM and MB performed B16 experiments. AB analyzed data, AB and MvdB interpreted data. HY and BB provided antibodies and IL-12Fc, respectively, and provided scientific input. AB and MvdB wrote the manuscript. All authors reviewed the manuscript.

Title: Effective immunotherapy of advanced cancer in mice

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One Sentence Summary: Treatment with anti-CD40 + IL-2 complexes + IL-12 promotes priming of tumor-specific $CD8^+$ T cells with sustained effector function, prevents induction of tolerance and has curative potential in mouse models of advanced cancer.

Abstract: Naïve T cells are insufficiently primed and become progressively dysfunctional in the context of cancer. Boosting antitumor responses by blocking PD-1 or CTLA-4 results in durable clinical responses only in a limited proportion of cancer patients suggesting that other pathways must be targeted to improve clinical efficacy. Our preclinical study identified anti-CD40 + IL-2 complexes + IL-12Fc as a uniquely efficacious treatment that prevents tolerance induction, promotes priming of sustained, protective tumor-specific $CD8^+$ T cells and cures late-stage cancer when given together with adoptively transferred tumor-specific T cells. We propose that improving signals 2 (costimulation) and 3 (cytokines) together with fresh tumor-specific rather than dysfunctional pre-existing memory T cells represents a potent therapy for advanced cancer.

6.1.1. Introduction

The immune system plays a significant role during tumorigenesis with evidence for a promoting as well as a suppressing role (*1*). Indeed, the amount and location of tumor-infiltrating Th1 and cytotoxic T cells is a strong positive prognostic factor in multiple types of human cancers (*2*). However, advanced tumors have intrinsic capacities to evade immune attack (immunoediting) as well as extrinsic mechanisms including a suppressive microenvironment, which also limits efficacy of conventional therapies (*1*).

The fact that radio- and chemotherapy can support tumor-specific immunity suggests that immune stimulation synergizes with such therapies (*3-5*). Multiple immunotherapeutic approaches such as vaccines (*6*), adoptive cell transfers (*7*), induction of antigen-presenting cell (APC) maturation (*8, 9*), checkpoint blockade (*10, 11*) and cytokines (*12, 13*) showed some success in boosting tumor-specific immunity both in mice and humans, although objective clinical responses were scarce. The main aim of immunotherapy is improving protective effector function of tumor-specific T cells, which relies on 3 signals: Stimulation of the TCR receptor (signal 1), costimulation (signal 2) and supportive cytokines (signal 3) (*14*). Immature dendritic cells (DCs) do not sufficiently provide signals 2 and 3 and induce robust, antigen-specific T cell tolerance (*15-17*). Although the requirements for T cell activation are well characterized, how to prevent tolerance in the context of cancer remains unclear.

We therefore aimed to identify a way to prime tumor-specific $CD8^+$ T cells, prevent induction of tolerance and achieve control of large, established tumors. We used TRAMP mice that develop autochthonous prostate cancer (*18*) combined with adoptive transfer of tumor-specific TCR transgenic $CD8^+$ T cells (TCR-I) (*19*) as well as advanced B16 melanoma. We found that the combination of agonistic anti-CD40 + IL-2/anti-IL-2 complexes (IL-2cx) + IL-12Fc was a distinctively effective treatment with respect to priming protective, tumor-specific immunity and eradicating tumors at advanced disease stage.

6.1.2. Results

Tumor-specific CD8$^+$ T cells are insufficiently primed in tumor-bearing mice

To study whether tumor-specific CD8$^+$ T cells are primed to develop full effector function in the context of a large, established tumor, we adoptively transferred CFSE-labeled tumor-specific CD8$^+$ T cells (TCR-I) into 13-14 weeks old male TRAMP (TG) mice and WT mice. TCR-I cells migrated into the prostate of TRAMP mice (Figures 1A and 1B) and proliferated in all organs analyzed as measured by CFSE dilution showing that TCR-I cells recognized their cognate antigen (Figure 1C). However, only a small proportion of TCR-I cells developed effector function as characterized by low IFN-γ production and degranulation (CD107a$^+$) (Figures 1D and 1E). Furthermore, IFN-γ production decreased over time, whereas the co-inhibitory molecule PD-1 increased (Figures 1D and 1F). Similar results were observed when TCR-I cells were transferred into TRAMP mice with an age ranging from 12 to 25 weeks (data not shown). Thus, in the context of cancer, CD8$^+$ T cells are inefficiently primed and progressively lose their already limited effector function.

Since proper T cell activation depends on antigen presentation by mature dendritic cells (DC) (*17, 20*), we tested whether insufficient DC maturation was responsible for poor CD8$^+$ T cell priming using co-injection of agonistic anti-CD40 antibodies. Eight days after adoptive transfer, spleen, prostate draining lymph nodes (PDLNs) and prostate were harvested and analyzed by flow cytometry (Figure 2A). Compared to controls, co-injection of anti-CD40 resulted in a 45- to 90-fold increase in TCR-I numbers in all organs analyzed, whereas the number of tumor-infiltrating endogenous CD8$^+$ T cells rose by 3-fold (Figure 2B). The frequency of IFN-γ producing cells was higher in the PDLNs and to some extent in the tumor (Figure 2C), as was the frequency of degranulating cells in the periphery (Figure 2D). Additionally, the amount of IFN-γ produced by TCR-I cells (IFN-γ GMFI of IFN-γ$^+$ cells) increased significantly in every organ analyzed (Figure 2C). Prostatic CD8$^+$ to regulatory T cell (CD45.2$^+$ CD4$^+$ FoxP3$^+$) ratio increased (Figure 2E) and PD-1 surface expression on TCR-I cells was reduced upon anti-CD40 treatment (Figure 2F). Anti-

CD40 treatment had an opposite effect on the PD-1 surface expression of endogenous $CD8^+$ T cells, which may indicate T cell activation. These data indicate that insufficient DC maturation precludes the development of effector function of tumor-specific $CD8^+$ T cells in TRAMP mice.

Naïve and effector tumor-specific $CD8^+$ T cells lose function in tumor-bearing mice

To investigate whether tolerance induction in the context of cancer is antigen-dependent, we co-transferred TCR-I and P14 cells (as control) into WT and TRAMP mice. All mice were subsequently infected with two recombinant vaccinia viruses, one coding for $SV40LT_{206-215}$ (rVV-I), the other for LCMV gp (rVV-G2), to trigger systemic responses in both transferred populations (Figure 3A). As expected, a high proportion of TCR-I and P14 cells in the spleen and PDLNs of WT mice produced IFN-γ. In contrast, responses of TCR-I, but not P14 cells, were significantly lower in TRAMP mice (Figures 3B and S1A-S1L), suggesting the induction of tumor-specific tolerance. PD-1 surface expression was elevated only on TCR-I cells of TRAMP mice, while P14 cells had similar levels of PD-1 in WT and TRAMP mice (Figure 3C). Thus, antigen encounter by $CD8^+$ T cells in the context of established cancer induces robust T cell tolerance, which cannot be overcome by subsequent viral challenge.

Because adoptive T cell therapy is a rapidly evolving and promising approach to treat multiple human cancer types (*7*), we adoptively transferred tumor-specific $CD8^+$ effector T cells, challenged mice 7 days later with rVV-I and assessed TCR-I function on day 12 (Figure 4A). Both in spleen and PDLNs of TRAMP mice, effector TCR-I cells displayed a 2-fold reduction of the frequency of IFN-γ^+ cells compared to WT mice and produced significantly less IFN-γ on a per-cell basis (Figure 4B). However, prior activation of TCR-I cells prevented PD-1 upregulation *in vivo* (Figure 4C) as compared to naïve cells (Figure 3C). These findings indicate that both naïve and effector tumor-specific $CD8^+$ T cells rapidly become functionally compromised in tumor-bearing mice although naïve T cells are more susceptible.

Preventing tolerance induction by immune intervention

The use of rVV-I infection is a robust read-out for tolerance. We used this set-up to identify interventions that prevent tolerance induction (Figure 5A). We tested combinations of different treatments such as complexes of recombinant murine IL-2 and S4B6 mAb (IL-2cx), recombinant murine IL-12 fused to murine IgG3 Fc (IL-12Fc), agonistic anti-CD40, blocking anti-CTLA-4 and anti-PD-1 mAbs. Data from 3 independent experiments were pooled and for purpose of comparison, we normalized the results to responses without intervention in WT (set to 100%) and TRAMP (set to 0%) (Figures S2A-S2C) for each of the 6 different read-outs used. Depending on the read-out, the individual treatments had different rankings (Tables S1, S2 and S3), suggesting that some interventions support proliferation and/or survival of TCR-I cells whereas others impact more on function, thus providing a rationale for combined use. Agonistic anti-CD40 was present in the 5 best treatments and was also the most potent single agent therapy, underscoring the importance of APC activation. The combination composed of IL-2cx + IL-12Fc + anti-CD40 was the most potent regimen tested overall (Figure S2C and Table S3) with respect to TCR-I numbers (Figure 5B), IFN-γ production (Figure 5C), degranulation (Figure 5D) and concomitant reduction in PD-1 surface expression (Figure 5E). Thus, simultaneous targeting of multiple pathways prevents the induction of tumor-specific $CD8^+$ T cell tolerance in tumor-bearing mice.

Treatment with anti-CD40 + IL-2cx + IL-12Fc turns tolerance of local and systemic tumor-specific immunity into priming

To investigate whether anti-CD40 + IL-2cx + IL-12Fc not only prevents tolerance induction but also promotes priming of tumor-specific $CD8^+$ T cells in tumor-bearing mice, we transferred naïve TCR-I cells into TRAMP mice and started therapy on the same day (Figure 6A). One week after adoptive transfer, we found a significant increase in absolute numbers of TCR-I cells and in the frequency and quality of IFN-γ producing TCR-I cells in the periphery as well as at the tumor site of treated mice (Figures 6B and S3A), while high surface PD-1 expression was prevented upon

treatment (Figure S3B). None of the other combinations tested was as efficient as anti-CD40 + IL-2cx + IL-12Fc (Figures S4A-S4F).

To investigate whether this therapy can rescue tolerized TCR-I cells, we started treatment 2 weeks after adoptive transfer and performed analysis a week later (Figure 6C). The combination of anti-CD40 + IL-2cx + IL-12Fc rescued tolerized TCR-I cells with respect to their numbers (Figure S3C), the frequency of IFN-γ producing TCR-I in the PDLNs and the per-cell production of IFN-γ in PDLNs and prostate (Figure 6D). A proportion of TCR-I cells in the prostate expressed lower levels of PD-1 in treated animals (Figure S3D).

To investigate whether this combination sustains long-term tumor-specific immunity, we treated adoptively transferred mice for a week and analyzed 2 weeks later (Figure 6E). Again, the treated group showed superior responses in terms of absolute numbers (Figure S3E), frequency and intensity of IFN-γ production (Figure 6F) and PD-1 expression (Figure S3F) by TCR-I cells. On day 21 after the start of the therapy, the treated mice had significantly reduced tumor burden compared to controls (Figures 6G and 6H). This reduction was not observed one week after treatment (prevention approach shown in Figure 6A) or when treatment was started 2 weeks after adoptive transfer (rescue approach shown in Figure 6C). Surprisingly, when combining the data from these 3 different experimental set-ups (Figures 6A, 6C and 6E), we found an inverse correlation between the logarithms of IFN-γ GMFI and PD-1 GMFI restricted to IFN-γ^+ TCR-I cells, with the strongest association ($R^2 = 0.9315$) in the prostate (Figure S5). Thus, treatment with anti-CD40 + IL-2cx + IL-12Fc leads to clinical responses with durable local and systemic immunity. Also, tolerized tumor-specific $CD8^+$ T cells were rescued to a limited extent, albeit without impact on tumor burden at the time point investigated.

To investigate the impact of the treatment on endogenous tumor-specific $CD8^+$ T cells, we used 3 well-characterized H-2^b-restricted CD8 epitopes derived from SV40LT (*21*). Mice were treated as described (Figures 6A and 6C) and single cell suspensions from spleen, PDLNs and prostate were stimulated *in vitro* with the 3 pooled SV40LT peptides, followed by intracellular

staining for IFN-γ. Seven days after transfer, the frequency of IFN-γ^+ endogenous $CD8^+$ cells was significantly higher in treated mice compared to controls (Figure 7A) as were absolute counts of endogenous $CD8^+$ T cells in the prostate (Figure 7B). However, this effect was short-lasting as it was not detected 2 weeks after cessation of therapy. Higher numbers of IFN-γ producing endogenous $CD8^+$ T cells correlated with higher surface PD-1 levels as compared to the control group (Figures 7C and S6), in contrast to the observations with TCR-I cells (Figures 6C and S4). The highest endogenous $CD8^+$ to Treg ratio was observed on day 21 (Figure 7D), but this was mainly due to the low number of Tregs in the prostate (Figure 7E). Because we only analyzed the response against three SV40LT-derived epitopes and ignored other potential tumor-specific antigens, we most likely underestimated the endogenous $CD8^+$ T cell response to the tumor. Thus, anti-CD40 + IL-2cx + IL-12Fc also enhance endogenous tumor-specific immunity.

Treatment with anti-CD40 + IL-2cx + IL-12Fc shows clinical efficacy in late-stage cancer models

We investigated the clinical efficacy of the therapy for advanced tumors using TRAMP mice and subcutaneously injected B16F10 syngeneic melanoma that does or doesn't express ovalbumin (OVA) (Figures 8A and 8E).

TRAMP mice were treated at the age of 20 weeks when all mice have prostatic intraepithelial neoplasia and 80% of mice show adenocarcinomas (*22*). While the control group had a median tumor-free survival of 32.3 weeks and median overall survival of 37.6 weeks, mice that received either TCR-I cells alone or TCR-I cells + anti-CD40 + IL-2cx + IL-12Fc had significantly prolonged survival (Figures 8B and 8C). Mice that received only TCR-I cells had a median tumor-free survival of 41.3 weeks (vs control, $p < 0.0001$) and a median overall survival of 49.1 weeks (vs control, $p = 0.0002$). Strikingly, 85% (11/13) of the mice receiving TCR-I cells + anti-CD40 + IL-2cx + IL-12Fc remained tumor-free and 100% were alive at 45 weeks of age (tumor-free and overall survival, $p < 0.0001$ vs control). Most importantly, tumor-free and overall survival of mice treated with TCR-I cells + anti-CD40 + IL-2cx + IL-12Fc were significantly improved compared to

adoptive transfer alone ($p = 0.0055$ and $p = 0.0374$, respectively). The combination therapy required concomitant adoptive transfer of TCR-I cells to have clinical efficacy in this late-stage therapeutic setting of autochthonous prostate cancer. A one-week treatment with anti-CD40 + IL-2cx + IL-12Fc with concomitant adoptive transfer of TCR-I cells induced long-lasting protective immunity, as transferred TCR-I cells were detectable in blood of treated mice more than 6 months after adoptive transfer, but not in mice receiving only TCR-I cells. Although comparison was not possible at this late timepoint, peripheral TCR-I cells remained functional in treated animals in terms of IFN-γ secretion and degranulation when restimulated with $SV40LT_{206-215}$ peptide (Figure 8D). These data indicate that the combination therapy synergized strongly with adoptive transfer of TCR-I cells to eradicate advanced stage cancer in TRAMP mice and provided protective, long-lasting tumor-specific immunity.

The treatment of C57BL/6 mice bearing B16F10 or B16F10-OVA tumors started on day 13 after tumor injection (tumor size ~40-50 mm^2), a time at which single reagents cannot control tumor growth (*23 and unpublished data*). The median survival of B16F10-bearing mice treated with anti-CD40 + IL-2cx + IL-12Fc nearly doubled (34 days) compared to the controls (18 days) (Figures 8F and S7A). In the case of B16F10-OVA tumors, some of the mice received adoptively transferred OT-I cells i.v. on day 13. Without anti-CD40 + IL-2cx + IL-12Fc, mice with B16F10-OVA tumors had a median survival time of 21 days whether they received OT-I cells or not. Treatment with anti-CD40 + IL-2cx + IL-12Fc + OT-I cells prolonged the median survival to 52 days and cured 43% (3/7) of the mice (Figures 8G and S7B). Tumor rejection was accompanied by vitiligo indicating a strong and sustained response against melanocytes that did not extend beyond the initial site of tumor injection (Figure 8H). In this model, anti-CD40 and IL-2cx were given i.p. and IL-12Fc intratumoral. When all components were given i.p. using a 100-fold higher dose of IL-12Fc, we observed no cure although the median survival significantly increased (35 days vs 24 days) (data not shown). Thus, supporting tumor-specific $CD8^+$ T cells on 3 levels (anti-CD40, IL-2 and IL-12) generates protective immunity that can eradicate advanced tumors.

6.1.3. Discussion

The immune system plays an essential role in controlling cancer cells (*1*). The clinical response to drugs that modulate the immune system such as blockade of CTLA-4 and PD-1 or its ligands further illustrates that the immune system can be employed in the therapy of cancer (*11, 24, 25*). However, only a fraction of the patients responds to such therapies, suggesting that additional pathways must be targeted to improve clinical efficacy.

We identified the combination of anti-CD40 + IL-2cx + IL-12Fc as uniquely efficacious in inducing protective, long-lasting tumor-specific immunity of adoptively transferred and endogenous tumor-specific $CD8^+$ T cells. This immune intervention had clinical efficacy in late stage preclinical cancer models and curative potential when combined with adoptive T cell transfer.

Our observation that without intervention, naïve TCR-I cells proliferate upon adoptive transfer into TRAMP mice but are subsequently tolerized suggests that signals 2 (costimulation) and 3 (cytokines) rather than signal 1 (TCR) are limiting. Previous work has highlighted the critical importance of the CD40-CD40L axis in preventing T cell tolerance (*20, 26*). Our study confirms this since the 5 best therapies identified here comprised agonistic anti-CD40, which improves signal 2 through induction of DC maturation.

A major result of concomitant TCR and CD28 signaling is the production of IL-2 by T cells. However, IL-2 production is transient and Tregs compete with activated T cells for IL-2 (*27*), which may result in death of the latter due to IL-2 deprivation (*28*). This may be relevant especially in the tumor microenvironment, which usually contains high numbers of Tregs. Specific support to effector T cells is possible through the use of IL-2cx, which target IL-2 to antigen-experienced $CD122^{high}$ (IL-2Rβ) T cells (*29*).

While anti-CD40 + IL-2cx may support priming and expansion of tumor-specific $CD8^+$ T cells in the context of advanced cancer, their differentiation into protective effectors requires additional signals such as IL-12 (signal 3) (*16, 30*). Accordingly, combining anti-CD40 and IL-2cx with IL-12Fc significantly improved the quality of tumor-specific T cells with respect to many

parameters. Altogether, our data are in line with recent work showing that T cell division destiny depends on the integrated quality of signals 1, 2 and 3 (*31*).

Although APC maturation resulting in appropriate costimulation and local production of cytokines is essential and usually sufficient to prevent the induction of peripheral T cell tolerance, the immunosuppressive tumor microenvironment poses an additional challenge. For example, local overexpression of PD-1 ligands may negatively impact on T cell function. PD-1 was originally described as an activation marker on T cells (*32*), but later data showed that PD-1 acts as a coinhibitory molecule (*33*) involved in peripheral T cell tolerance (*34*) that is often used as a marker for exhausted T cells (*35*). TCR-I cells progressively upregulate the expression of PD-1 upon adoptive transfer into TRAMP mice, unless they are treated with immunostimulatory drugs. PD-1 expression on TCR-I cells correlates negatively with IFN-γ production, in agreement with PD-1 being a marker for T cell exhaustion/dysfuntion. However, endogenous $CD8^+$ T cells showed a different behavior with respect to PD-1 expression: Treatment with anti-CD40 + IL-2cx + IL-12Fc resulted in increased IFN-γ production and PD-1 expression. Because the levels of PD-1 on endogenous $CD8^+$ T cells in TRAMP mice treated with anti-CD40 + IL-2cx + IL-12Fc is substantially lower than that on TCR-I cells in untreated TRAMP mice, we propose that intermediate levels of PD-1 mark T cell activation, whereas very high levels indicate exhaustion/dysfunction supporting the view of PD-1 as a rheostat of immune responses (*36*). The apparently greater tendency of TCR-I cells to express very high levels of PD-1 in the context of advanced cancer when compared to endogenous $CD8^+$ T cells may be related to TCR affinity (*37*). All interventions tested here had an impact on PD-1 expression, although to various extent, pointing to PD-1 as a central player in the negative feedback mechanism following activation. However, because not all interventions improved T cell responses, blocking PD-1 is not sufficient to sustain protective immunity.

One of the biggest challenges in the field remains to reach maximal efficacy with manageable toxicity. Despite the fact that the combination of anti-CD40, IL-2cx and IL-12Fc was

well tolerated in mice, these reagents have severe side-effects at high doses in humans (*38, 39*). Such toxicities have limited the development of many candidate drugs in the past, but recently, less toxic molecules have been engineered and made their way into clinical trials. For example, the fusion of IL-12 to a tumor-specific antibody targeting it to necrotic regions of tumors showed promising efficacy in mice and better toxicity profile in cynomolgus monkeys than recombinant IL-12 (*40*). Other examples include site-specific mutations of IL-2 altering its affinity towards IL-2Rβ and losing the requirement for CD25 involvement in order to increase its activity on cytotoxic T cells and reduce it on regulatory T cells (*41*) or the use of IL-2cx as we did here, which have a better efficacy with reduced toxicity than recombinant IL-2 (*29, 42*).

Recent studies suggested *de novo* priming towards tumor-specific mutant antigens in the context of checkpoint blockade as important predictors of response to immunotherapy (*43-45*). Our study supports the importance of *de novo* priming and showed that rescuing pre-existing tumor-infiltrating $CD8^+$ T cells is inefficient. Along this line, the relatively long lag phase before immunotherapy is effective may be explained by dependence on *de novo* priming: pre-existing immunity is presumably compromised and cannot be sufficiently boosted. Instead, efficacy may rely on new thymic emigrants (or adoptively transferred T cells) that encounter tumor-specific antigens in an immunogenic context, which can be achieved by targeting multiple pathways involved throughout T cell priming and differentiation. Indeed, our data show that treatment with anti-CD40 + IL-2cx + IL-12Fc during the first week of adoptive transfer results in clinical efficacy and maintenance of protective effector TCR-I cells over a period of at least 6 months in TRAMP mice with advanced cancer. One can speculate that the favorable effects of lympho- and myeloablation prior to adoptive transfer of tumor-specific T cells may enhance *de novo* priming because immunosuppressive and compromised cells are deleted (*46*).

In conclusion, we have identified a novel therapeutic intervention for late-stage tumors that supports systemic and local tumor-specific immunity in a durable fashion and shows clinical responses. The efficacy of anti-CD40 + IL-2cx + IL-12Fc significantly outperformed all other

treatments we tested here and we think that combined improvement of signal 2 and 3 explains this result. To our knowledge, the combined therapy described here is the first example of successful curative treatment of late stage (day 13) B16F10 melanoma. Furthermore, our study shows survival benefit in the autochthonous and clinically relevant TRAMP model treated at an advanced disease stage (20 weeks) with only a single cycle of treatment with protective immunity lasting over 6 months following transfer.

We propose that an optimal cancer immunotherapy based on T cells should tackle 4 major obstacles. 1) The frequency of fresh tumor-specific T cells needs to be increased and adoptive transfer or cancer vaccines may represent optimal ways of achieving this goal. 2) Appropriate costimulation is essential. 3) Survival and differentiation of tumor-infiltrating T cells must be supported by local cytokine therapy. 4) Innovative delivery systems and careful dosage will be key enabling factors to limit treatment-related toxicities and achieve maximal efficacy.

Materials and Methods

Please refer to Supplementary Materials and Methods.

List of Supplementary Materials

Supplementary Materials and Methods

Fig. S1. Tolerization of TCR-I cells in TRAMP mice

Fig. S2. Preventing tolerance induction by immune intervention.

Fig. S3. Administration of anti-CD40 + IL-2 complexes + IL-12Fc improves local and systemic tumor-specific immunity.

Fig. S4. Immune interventions can shift the balance from tolerance into priming of tumor-specific immunity.

Fig. S5. Inverse correlation between PD-1 surface expression level and IFN-γ production by IFN-γ^+ TCR-I cells.

Fig. S6. Immune interventions trigger tumor-specific endogenous $CD8^+$ T cell responses.

Fig. S7. Tumor growth curves of subcutaneous B16 melanoma.

Fig. S8. Gating strategy for flow cytometry analysis.

Tables S1-S3. Impact of various immune interventions on preventing tolerance while improving proliferation and function of TCR-I cells (Data for spleen, PDLNs and summary, respectively).

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6.1.5. Figures and legends

Fig. 1

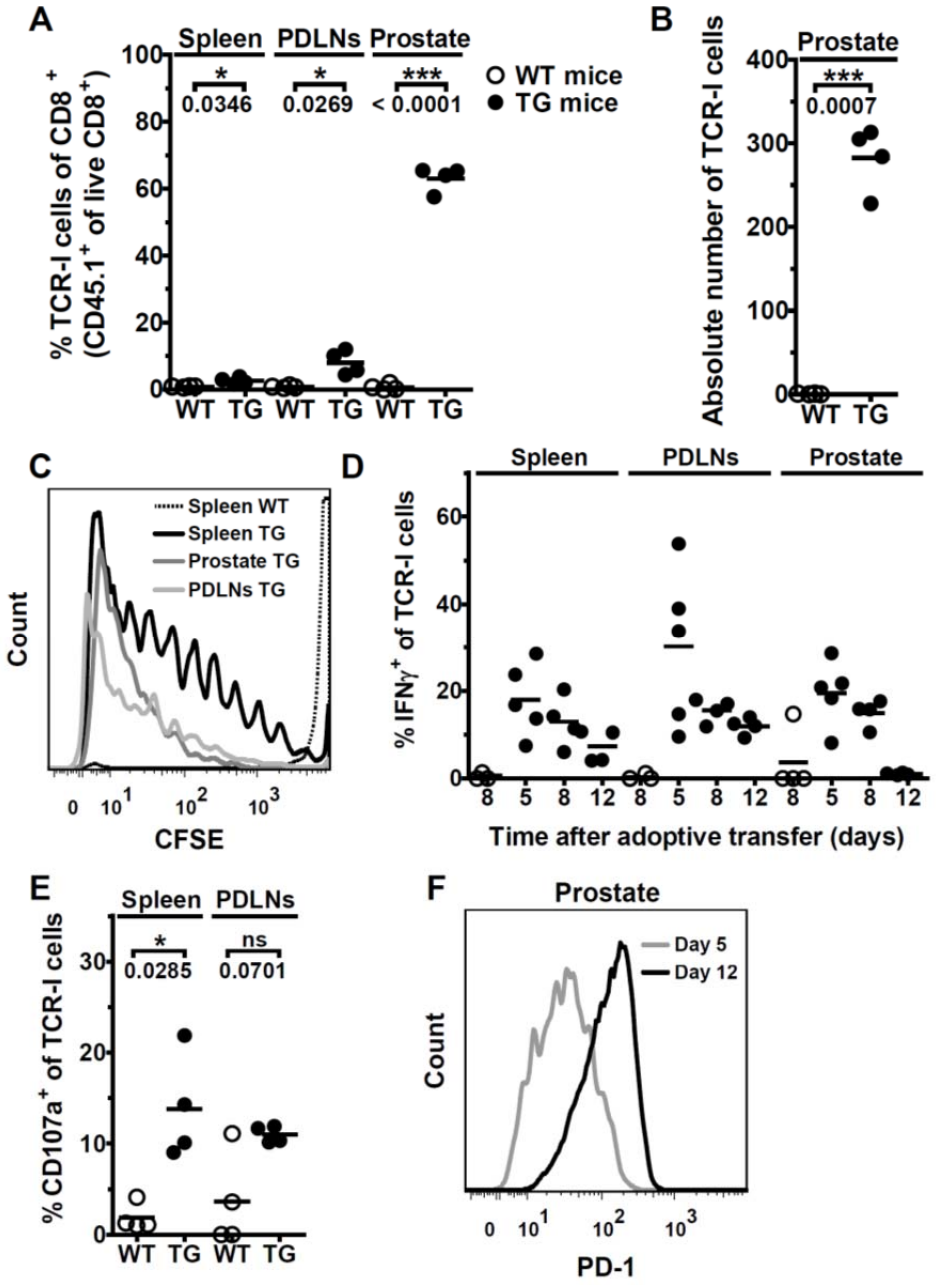


Figure 1. Tumor-specific TCR-I CD8$^+$ T cells proliferate and migrate to the tumor but do not develop effector function. One million CD8$^+$ TCR-I cells were adoptively transferred into 13-20 weeks old male TRAMP or WT mice. (**A**) The percentage of TCR-I cells of CD8$^+$ T cells in spleen, prostate-draining lymph nodes (PDLNs) and prostate 7 days after transfer. (**B**) Absolute number of CD8$^+$ CD45.1$^+$ TCR-I cells of live singlets per mg prostate 7 days after transfer. (**C**) Proliferation of TCR-I cells as measured by CFSE dilution in spleen, PDLNs and prostate 5 days after transfer. Two representative mice out of five are shown. (**D**) IFN-γ production in spleen, PDLNs and prostate by TCR-I cells at different time points after transfer. (**E**) Degranulation of TCR-I cells in spleen and PDLNs 8 days after transfer. (**F**) PD-1 surface expression on TCR-I cells in the prostate 5 and 12 days after transfer. Two representative mice of 9 are shown. WT, open symbols (○); TRAMP, closed symbols (●). Symbols represent individual mice (n=4-5 per group) and data from one out of two independent experiments are shown. Horizontal lines represent mean values.

Fig. 2

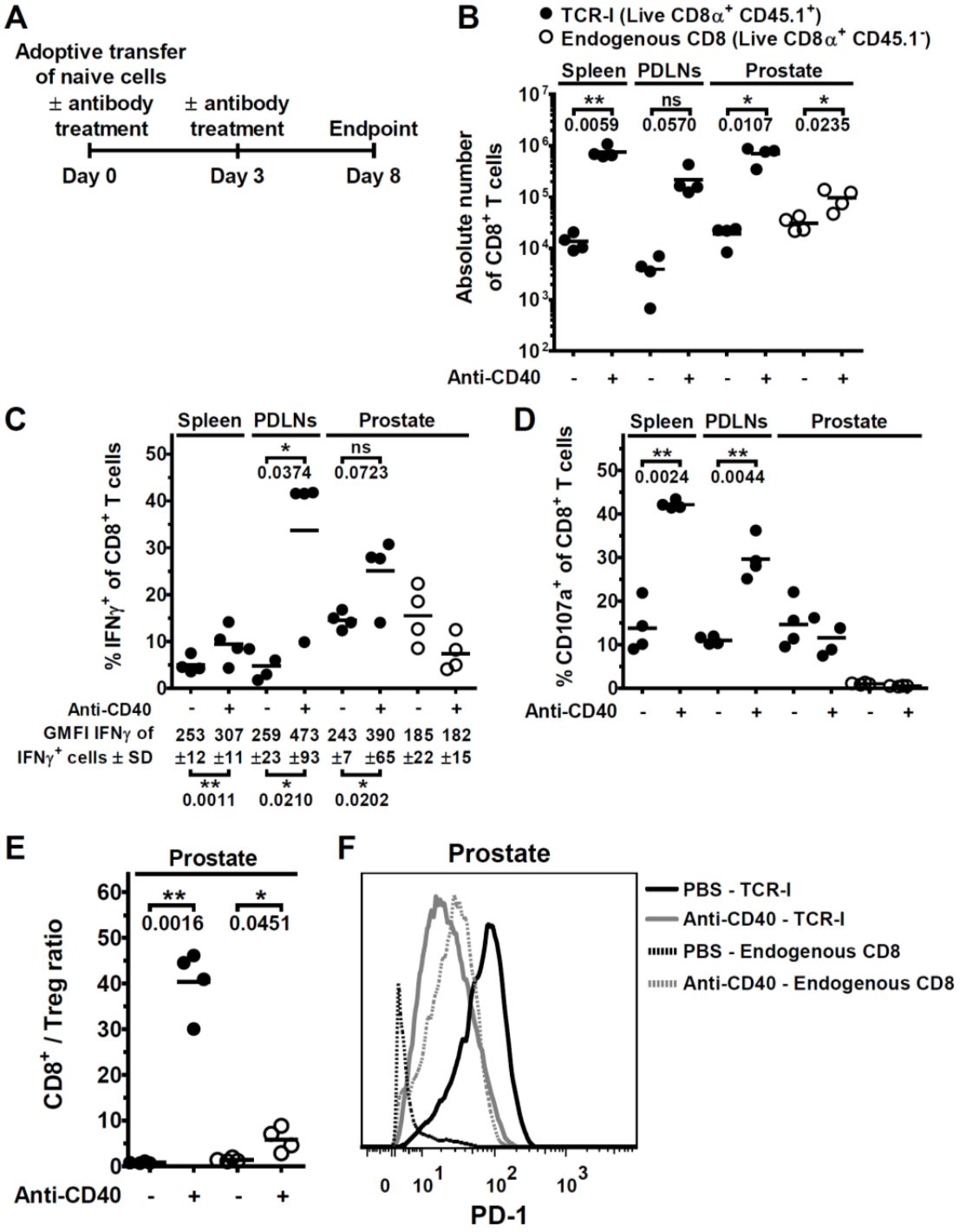


Figure 2. Agonistic anti-CD40 supports effector function of adoptively transferred tumor-specific TCR-I cells. (**A**) One million TCR-I cells were adoptively transferred into 11-14 weeks old male TRAMP mice and anti-CD40 was administered as depicted. (**B**) Absolute number, (**C**) IFN-γ production and (**D**) degranulation of TCR-I and endogenous $CD8^+$ cells in spleen, PDLNs and prostate 8 days after transfer. (**E**) Ratio of absolute number of $CD8^+$ to Treg and (**F**) PD-1 surface expression of two representative mice is illustrated. Endogenous $CD8^+$, open symbols (○); TCR-I, closed symbols (●). Symbols represent individual mice and data from one out of three experiments are shown. Horizontal lines represent mean values.

Fig. 3

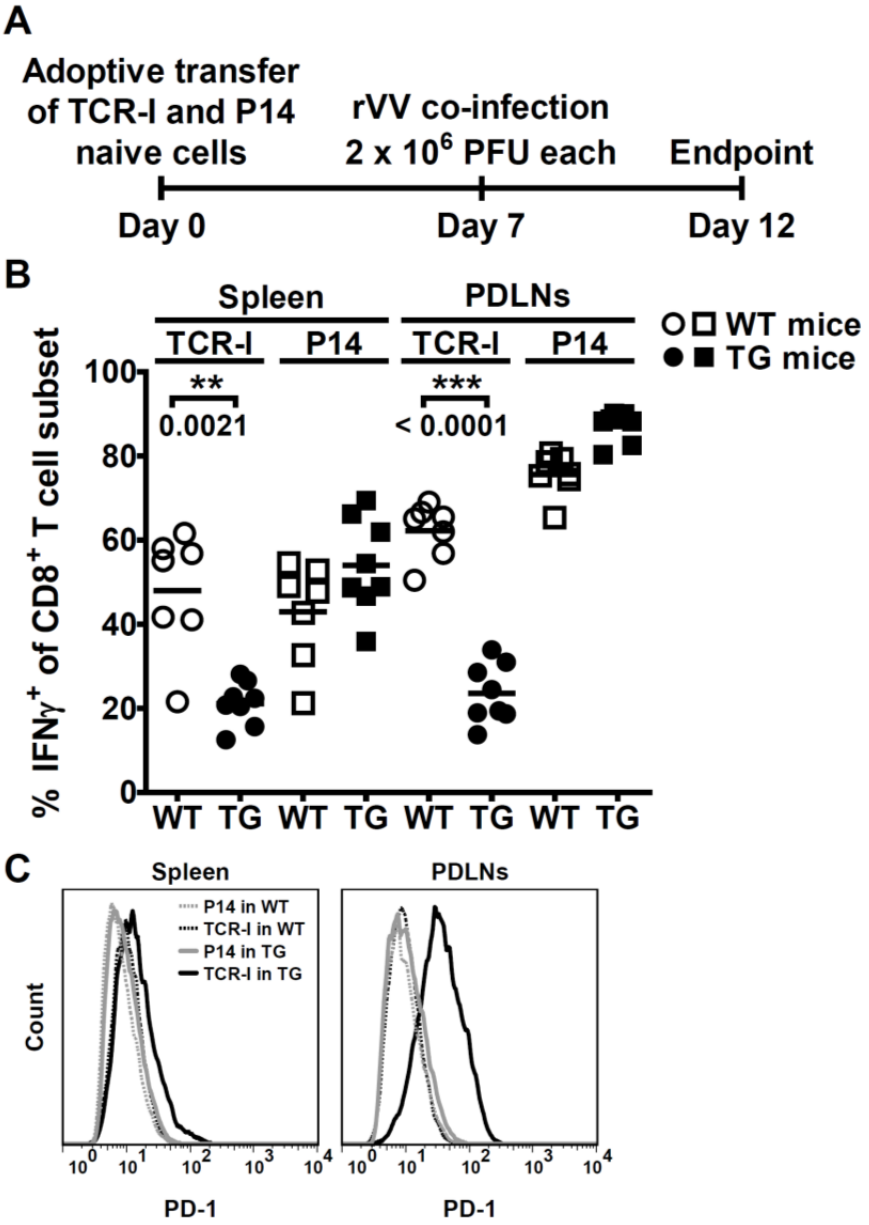


Figure 3. Tumor-specific naïve CD8$^+$ T cells are tolerized in TRAMP mice. (**A**) One million TCR-I (CD45.1$^+$2$^-$) + one million P14 (CD45.1$^+$2$^+$) TCR transgenic CD8$^+$ cells were adoptively transferred into 14-15 weeks old male TRAMP or WT mice. Mice were infected 7 days after adoptive transfer with a mixture of 10^6 PFU rVV-I + 10^6 PFU rVV-G2. TCR-I and P14 T cells were analyzed 5 days after infection. (**B**) IFN-γ production and (**C**) representative examples for PD-1 surface expression of TCR-I and P14 cells in the spleen and PDLNs 5 days post-infection. TCR-I cells, circles (○, ●); P14 cells, squares (□, ■). WT, open symbols (○, □); TRAMP closed symbols (●, ■). Groups were composed of 7 WT and 8 TRAMP mice and data from one out of two independent experiments are shown. Horizontal lines represent mean values.

Fig. 4

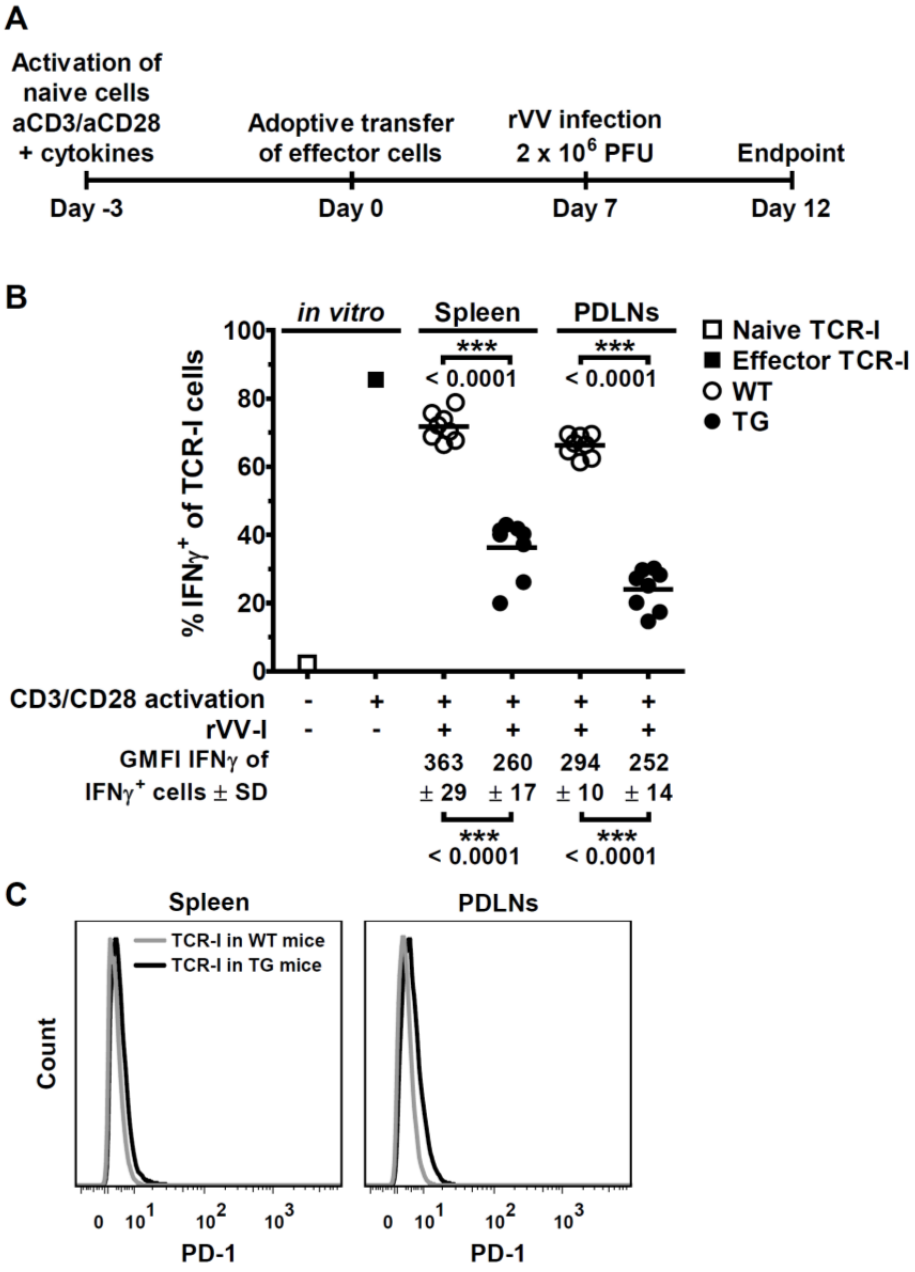


Figure 4. Tumor-specific, effector CD8$^+$ T cells are tolerized in TRAMP mice. (**A**) Five million CD8$^+$ TCR-I effector cells were adoptively transferred into 12-14 weeks old male TRAMP or WT mice that were infected 7 days later with 2 x 10^6 PFU rVV-I. (**B**) Frequency and intensity of IFN-γ production by CD8$^+$ TCR-I cells in spleen and PDLNs are shown 5 days post-infection. (**C**) Representative examples of PD-1 surface expression of TCR-I cells at the same time point. WT, open circles (○); TRAMP, closed circles (●). Circles represent individual mice (n=8 per group) while squares (□, ■) represent the values obtained from *in vitro* culture of CD8$^+$ TCR-I cells. Data from one out of two independent experiments are shown. Horizontal lines represent mean values.

Fig. 5

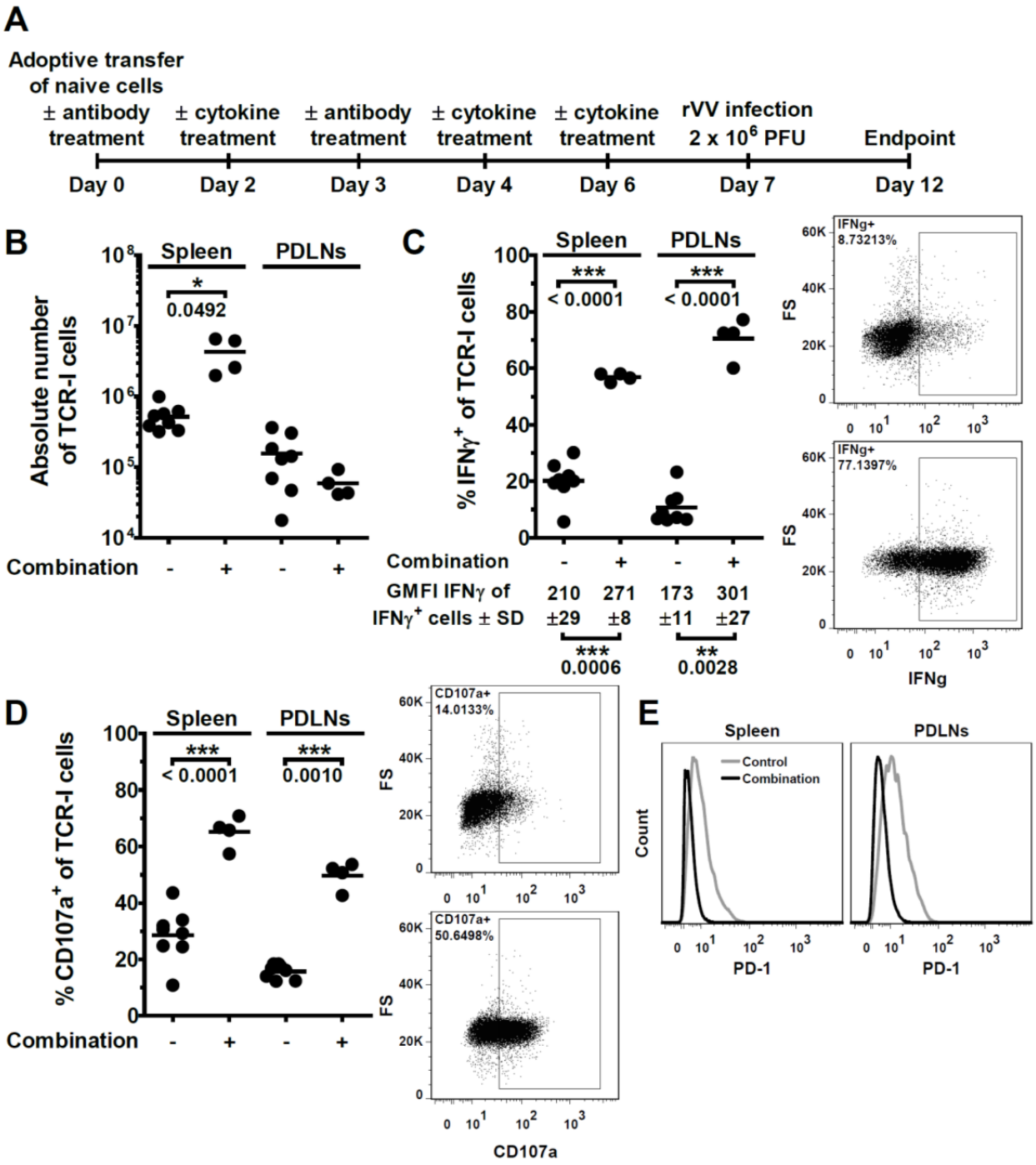


Figure 5. Prevention of tolerance by anti-CD40 + IL-2cx + IL-12Fc. (**A**) One million CD8$^+$ TCR-I cells were adoptively transferred into 13-16 weeks old male TRAMP or WT mice that received (or not) anti-CD-40 + IL-2cx + IL-12. Mice were infected 7 days after adoptive transfer with 2 x 10^6 PFU rVV-I. TCR-I cells were analyzed 5 days after infection. (**B**) Absolute number, (**C**) IFN-γ production and (**D**) degranulation of TCR-I CD8$^+$ cells in spleen and PDLNs are shown 5 days post-infection. (**E**) PD-1 surface expression of two representative mice is illustrated. Symbols represent individual mice (n=4-8 per group) and data from one out of three independent experiments are shown. Horizontal lines represent mean values.

Fig. 6

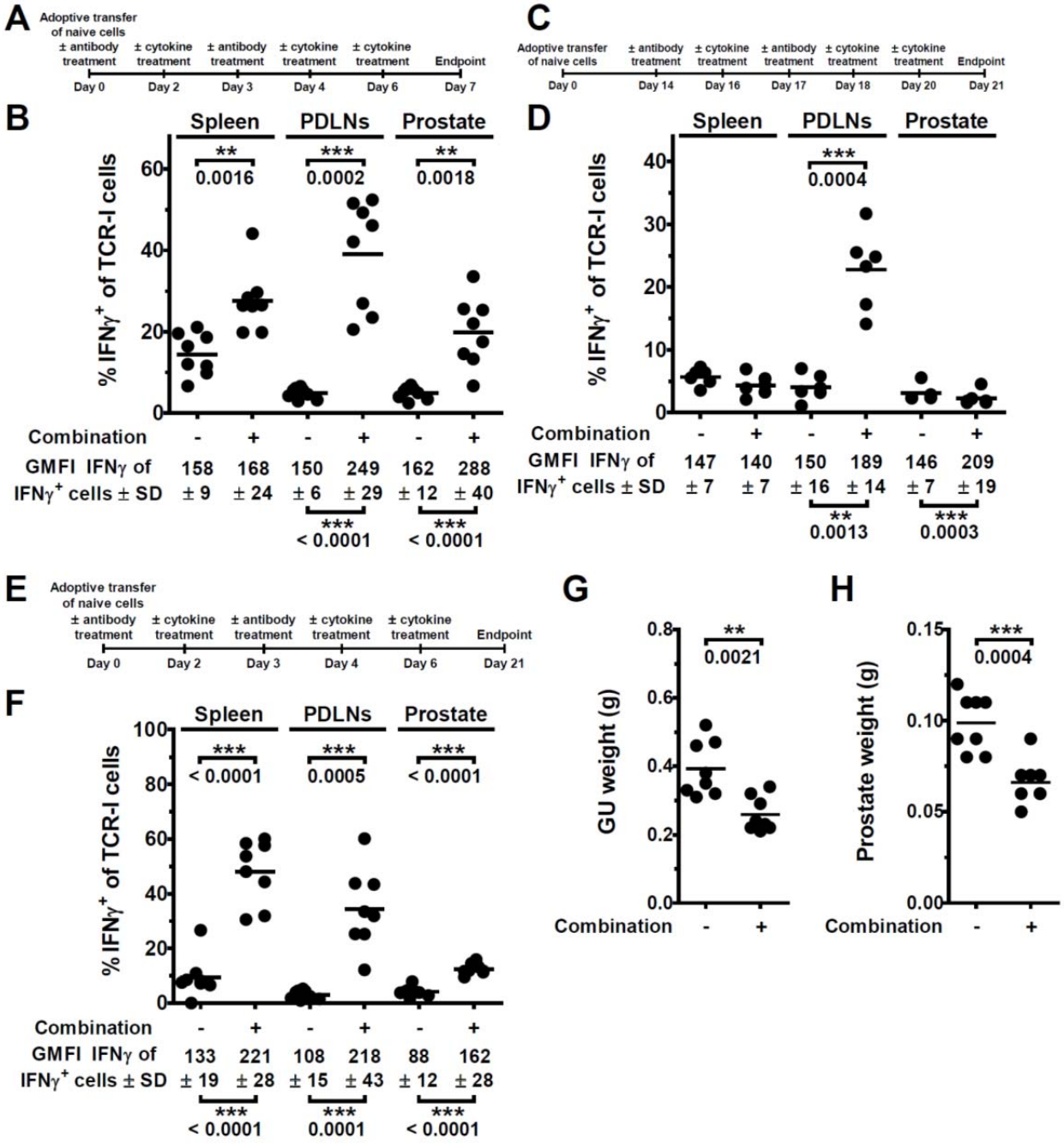


Figure 6. Administration of anti-CD40 + IL-2cx + IL-12Fc improves local and systemic tumor-specific immunity. One million naïve TCR-I cells were adoptively transferred into 16-18 weeks old male TRAMP mice. (**A**) Treatment schedule for the prevention of tolerance induction in TCR-I cells. (**B**) Frequency and intensity of IFN-γ production by TCR-I cells. (**C**) Treatment schedule to investigate the rescue of tolerized TCR-I cells. (**D**) Frequency and intensity of IFN-γ production by TCR-I cells in the spleen, PDLNs and prostate of TRAMP mice. (**E**) Treatment schedule to examine sustained responses by TCR-I cells. (**F**) Frequency and intensity of IFN-γ production by TCR-I cells. (**G**) Weight of the genitourinary tract (seminal vesicles + prostate gland only) at the endpoint of the experiment assessing sustained responses. (**H**) Weight in grams on day 21 of dissected prostate gland. Symbols represent individual TRAMP mice and data from three out of seven independent experiments are shown. Horizontal lines represent mean values.

Fig. 7

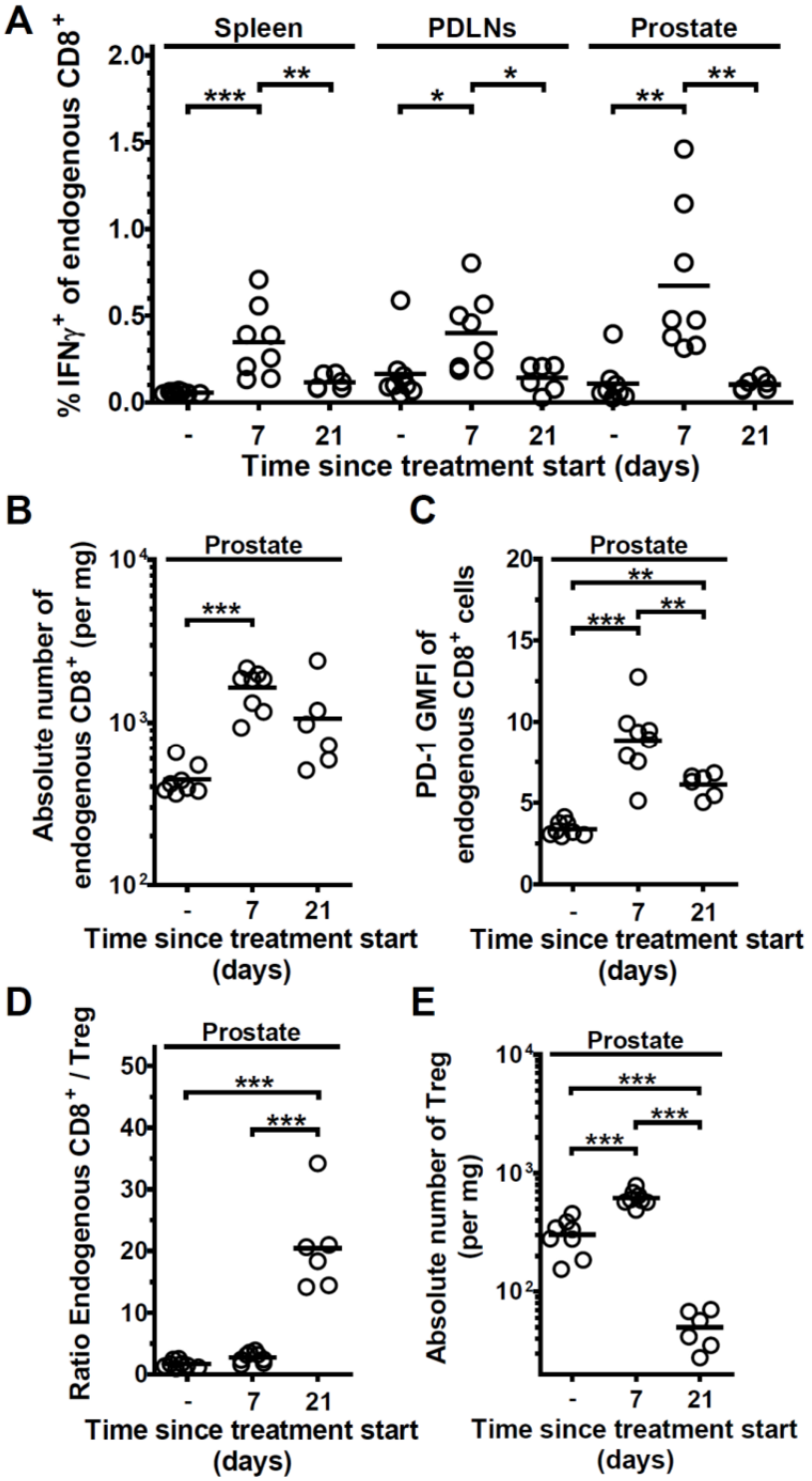


Figure 7. Administration of anti-CD40 + IL-2cx + IL-12Fc improves endogenous tumor-specific immunity. Endogenous $CD8^+$ $CD45.1^-$ cells were analyzed following the experimental designs described in figures 6A and 6D. Cells were stimulated for 5 hours in presence of a combination of three different SV40LT-derived peptides. (**A**) Frequency of IFN-γ production by endogenous $CD8^+$ T cells at different time points after treatment. (**B**) Absolute number and (**C**) surface PD-1 expression of endogenous $CD8^+$ $CD45.1^-$ cells. (**D**) Ratio of absolute number of $CD8^+$ $CD45.1^-$ cells to $FoxP3^+$ $CD4^+$ Treg cells. (**E**) Absolute number of $FoxP3^+$ $CD4^+$ Treg cells in the prostate. Symbols represent individual TRAMP mice and data from one out of two independent experiments are shown. Statistics were made by one-way ANOVA and Tukey-Kramer post-test to compare all pairs per organ. Horizontal lines represent mean values.

Fig. 8

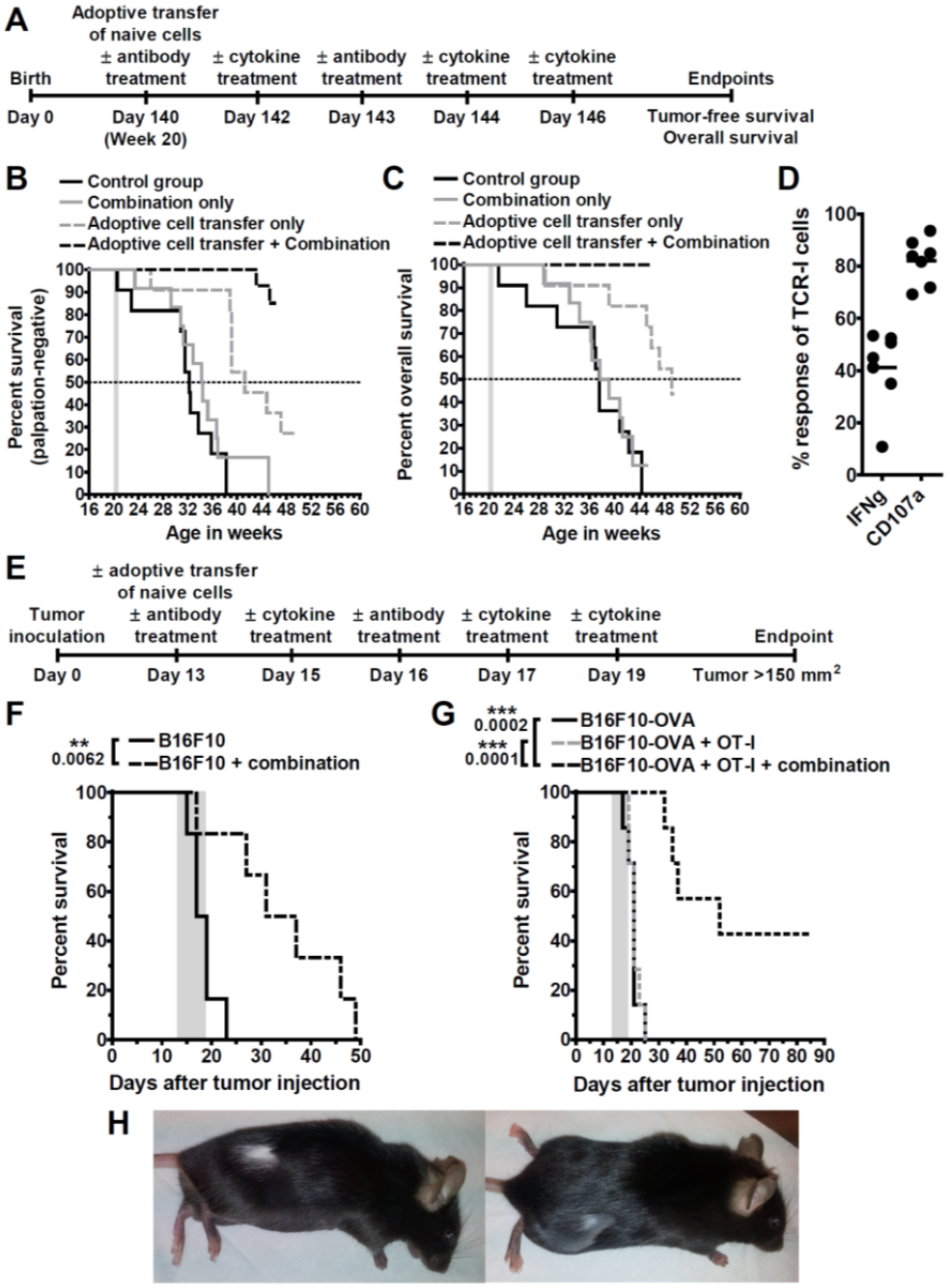


Figure 8. Administration of anti-CD40 + IL-2cx + IL-12Fc has clinical efficacy in late-stage cancer. (**A**) Treatment schedule for TRAMP mice. (**B**) Tumor-free survival of TRAMP mice. Event: tumor of ≥ 1 cm^3. (**C**) Overall survival of TRAMP mice receiving different treatments. The event is defined as one out of 5 following criterias: lack of escape behavior, poor general condition, hunched back, ruffled fur or abdominal distension. (**D**) Frequency of IFN-γ and CD107-positive TCR-I cells 6 months after adoptive transfer in treated animals. (**E**) Experimental design for late-stage treatment of subcutaneous B16 melanoma. Event: tumor size reaching 150 mm^2 (length x width). (**F**) Kaplan-Meier survival analysis of mice injected with B16F10 tumors. (**G**) Kaplan-Meier survival analysis of mice bearing B16F10-OVA tumors, some of which received one million naïve OT-I $CD8^+$ cells. (**H**) Pictures on day 99 of two out of three cured mice showing depigmentation at the site of the rejected tumor. Gray shades on graphs illustrate the 7 days treatment course. TRAMP survival analysis was performed once (n=11-14 mice per group). Data from two out of four experiments are shown for the subcutaneous models (n=7 mice per group). Log-rank (Mantel-Cox) statistical tests were performed between pairs as described.

6.1.6. Supplementary Materials and Methods

Mice

TRAMP on a pure C57BL/6 background (*18*) and 416Tev/J (*19*) mice were purchased from the Jackson Laboratory. P14 mice (*47*) and OT-I mice (*48*) were obtained from the Laboratory Animal Service Center (University of Zurich). C57BL/6JOlaHsd mice were obtained from Harlan Laboratories. 416Tev/J mice carry a rearranged TCR (TCR-I) specific for the H2-D^b-restricted SV40LT epitope I (aa 206-215). P14 mice carry a rearranged TCR specific for a H2-D^b-restricted epitope derived from amino acids 33-41 of lymphocytic choriomeningitis virus glycoprotein (LCMV gp). OT-I mice carry a rearranged TCR specific for a H2-K^b-restricted epitope derived from amino acids 257-264 of ovalbumin (OVA). TRAMP females were maintained homozygous and bred with C57BL/6 males to generate heterozygous TRAMP males for experiments. Age- and sex-matched non-transgenic littermates or C57BL/6 were used as WT controls. TCR transgenic mice were maintained heterozygous and bred to carry the congenic marker CD45.1 (Ly5.1). All mice were kept under specific pathogen-free conditions in the facilities of the Institute of Laboratory Animal Science (University Hospital Zurich) and had access to standard chow (Provimi Kliba Cat.No. 3436) and water *ad lib*. Experiments were performed in accordance with the Swiss federal and cantonal regulations on animal protection and were approved by the Swiss cantonal veterinary office (Zurich).

Tumor models

TRAMP mice carry the oncogenic SV40 large T antigen (SV40LT) as a transgene under the control of the hormone-dependent, prostate-specific rat probasin promoter, resulting in the development of progressive prostate cancer in male mice. At 8 weeks of age, 100% of mice have prostatic intraepithelial neoplasia. By 12 weeks of age, all mice have adenocarcinomas which progresses gradually to large poorly differentiated tumors. Metastases occur in TRAMP mice with an incidence of ~50% by 24 weeks of age (*22*). For survival experiments, body weight measurements and

abdominal pelvic palpations were performed every 1-2 weeks from the age of 20 weeks. Mice with palpable tumors at 20 weeks were excluded from the study. The event in tumor-free survival was defined as the detection of a tumor >1 cm^3 by palpation. Death event for overall survival was defined as at least one out of five of the following termination criteria: lack of escape behavior, poor general condition, hunched back, ruffled fur or abdominal distension. B16F10 melanoma cells (ATCC CRL-6475) and B16F10-OVA (B16F10 stably transfected to express chicken ovalbumin as neo-antigen), kindly provided by Melody Swartz, EPFL, Lausanne, Switzerland, were cultured in Dulbecco's modified Eagle's medium (GIBCO Invitrogen) supplemented with 10% fetal calf serum, 0.1 mM 2-mercaptoethanol, 2 mM L-glutamine and antibiotics. To establish syngeneic tumors, 2 x 10^5 cells were injected s.c. in 100 µL PBS. Tumors were measured with a caliper every 2-3 days in two dimensions (length and width) and a death event was defined as tumor size reaching 150 mm^2 (length x width). Mice were randomized in order to have equal average tumor size and minimal standard deviation differences between groups on the day before starting therapy.

Adoptive Transfer of $CD8^+$ T cells

$CD8^+$ cells were isolated from spleens of TCR-I or P14 TCR transgenic mice by positive selection using CD8α MicroBeads according to manufacturer's protocol (Miltenyi Biotec). To track cell divisions, $CD8^+$ T cells were incubated for 10 minutes at 37ºC with 1 µM carboxyfluorescein diacetate succinimidyl ester (CFSE) (Sigma-Aldrich) in PBS, washed with media containing 10% fetal bovine serum (Gibco) and resuspended in PBS before injection. To generate effector $CD8^+$ T cells, $CD8\alpha^+$ cells were cultured for 3 days in RPMI-1640 supplemented with 1x MEM non-essential amino acids, 10 mM HEPES, 50 U/mL penicillin, 50 µg/mL streptomycin, 2 mM L-glutamine, 10% fetal bovine serum and 0.1 mM 2-mercaptoethanol (Gibco) in the presence of Dynabeads® Mouse T-Activator CD3/CD28 (Life technologies) with a bead-to-cell ratio of 1:1 and recombinant murine cytokines as follows: 60 IU/ml IL-2, 1 ng/mL IL-7, 10 ng/mL IL-15 and 10

ng/mL IL-21 (Peprotech). Unless otherwise stated, each mouse received 10^6 $CD8^+$ T cells by i.v. tail vein injection.

Treatment of tumor-bearing mice

The following monoclonal antibodies were used for treatment of TRAMP mice: anti-CD40 (FGK45), anti-CTLA-4 (UC10-4F10-11) and anti-PD-1 (RMP1-14). Antibodies were purified from culture supernatant using protein G sepharose 4 Fast Flow (GE Healthcare) columns according to manufacturer's protocol. Antibodies were administered at 50 µg (anti-CD40) or 250 µg (all others) in 200 µl PBS. IL-2/anti-IL-2 complexes were prepared with the S4B6 mAb as described (*29*) and an equivalent of 15000 units of recombinant mouse IL-2 (eBioscience) was used per injection. Recombinant mouse IL-12Fc was produced as described (*49*) and bioactivity was quantified by an IFN-γ secretion assay with ConA-stimulated splenocytes as previously described (*50*) using the following formula: specific activity (units/mg) = 10^6 / ED_{50} (ng/ml). IL-12Fc was administered in PBS at 10000 units/kg per i.p. injection for TRAMP mice or 100 units/kg per i.t. injection for subcutaneous models. All other substances were injected i.p. at time points indicated in the figures.

Generation of recombinant vaccinia virus and infection

Vaccinia virus (VV), strain WR, was originally obtained from Dr. B. Moss (National Institutes of Health, Bethesda, MD, USA). Recombinant VV carrying LCMV gp (rVV-G2) was originally obtained from Dr. D. Bishop (Institute of Virology, Oxford, UK). Recombinant VV expressing the SV40LT epitope epitope I (aa 206-215) was generated as previously described (*51*). Briefly, the vector pSC11.30R.2 (*52*) was digested with the restriction enzymes NcoI and BglII. Two oligonucleotides were annealed (Fwd: 5'-CAT GTC CGC CAT CAA CAA TTA CGC CCA GAA GCT GTA-3', Rev: 5'-GAT CTA CAG CTT CTG GGC GTA ATT GTT GAT GGC GGA-3') and directly ligated to the digested pSC11.30R.2. This transfer vector was then used to generate a recombinant vaccinia virus coding for $SV40LT_{206\text{-}215}$ through homologous recombination at the

thymidine kinase locus during concomitant transfection and infection of human thymidine kinase deficient 143B cell line (ATCC CRL-8303) with VV WR. Recombinant viruses were selected using 5-bromo-2'-deoxyuridine (BrdU) and recombinant plaques were identified by β-galactosidase activity as previously described (*53*). Recombinant VV were plaque purified three times on BSC40 cells (ATCC CRL-2761). Viral titers were determined by the same method. All VV were propagated on BSC40 cells at a multiplicity of infection of 0.1. For infections, mice received 2 x 10^6 plaque-forming units i.p.

Peptides

$SV40LT_{206-215}$ (SAINNYAQKL), $SV40LT_{223-231}$ (CKGVNKEYL), $SV40LT_{404-411}$ (VVYDFLKC) and LCMV gp_{33-41} (KAVYNFATC) were purchased from PolyPeptide Laboratories in immunograde quality. Stocks of 10 mM were prepared in DMSO and stored at -20°C.

Flow cytometry

To obtain single-cell suspensions, spleens and lymph nodes were mechanically homogenized with a syringe plunger on a sterile filter. Prostates were cut into small pieces and digested for 1 hour at 37ºC with agitation in RPMI containing 1 mg/ml collagenase IV and 2.6 µg/ml (6 U/ml) DNase I (Sigma-Aldrich), washed once with RPMI followed by filtration through 40 µm cell strainer. For subsequent staining of surface markers, cells were washed and resuspended in PBS and stained for 20 min at room temperature with panels of fluorochrome-labeled antibodies. To detect intracellular IFN-γ, cells were restimulated for 5 hours at 37ºC with 10 µM of the relevant peptide in presence of 10 µg/ml brefeldin A (Sigma-Aldrich). In some cases, 2.5 µg/ml of FITC anti-mouse CD107a (clone 1D4B, BioLegend) was added during restimulation allowing detection of degranulation. After 5 hours, cells were washed in PBS followed by surface staining for 20 minutes at room temperature, washed again and fixed with 4% paraformaldehyde in PBS. Cells were washed with permeabilization buffer (20 mM EDTA + 2% FBS + 0.03% NaN_3 + 0.1% saponin in PBS). Samples

were incubated overnight at 4ºC with APC anti-mouse IFN-γ (clone XMG1.2, BioLegend). For staining of regulatory T cells (Treg), APC anti-mouse FoxP3 (clone FJK-16s) and the Foxp3 Transcription Factor Staining Buffer Set (eBioscience) were used according to manufacturer's protocol. Unless otherwise stated, the following antibodies were purchased from BioLegend: anti-CD45.1 (A20), anti-CD45.2 (104), anti-CD8α (53-6.7), anti-CD8β (53-5.8), anti-TCR Vβ7 (TR310), anti-TCR Vα2 (B20.1, Becton Dickinson), anti-CD4 (RM4-5), anti-CD25 (PC61), anti-PD-1 (RMP1-30 and 29F.1A12). Doublets were excluded based on linear and area values of the forward scatter. Dead cells were excluded using the live/dead® Fixable Violet Dead Cell Stain Kit (Life technologies). The detailed gating strategies are illustrated in Figure S8. Absolute counts were determined with CountBright™ Absolute Counting Beads from Life Technologies and used to calculate $CD8^+$ to Treg ($CD4^+$ $FoxP3^+$) ratios. Samples were measured using a CyAn ADP 9 flow cytometer (Beckman Coulter) and analyzed with FlowJo v7.6.5 software (Tree Star).

Statistical analysis

Statistical analyses were performed with GraphPad Prism version 5.03 (GraphPad Software, Inc). For comparisons, unless otherwise stated, unpaired, two-tailed Student's t tests with systematic Welch's correction were done with an alpha of 0.05. For comparison of more than 2 experimental groups, one-way ANOVA either with Tukey-Kramer's post-test was used to compare all pairs or Dunnett's post-test to compare all groups to the control as mentioned in figure legends. A log-rank test (Mantel-Cox) was used for Kaplan-Meier survival curves comparison between selected pairs. P-values < 0.05 were considered statistically significant and marked with asterisks (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$). In Figure S2, two outliers identified with a significant Grubb's test (alpha 0.05) with the online tool (http://graphpad.com/quickcalcs/Grubbs1.cfm) are shown in the bar graph, but were excluded from statistical analysis.

6.1.7. Supplemental Figures and Legends

Fig. S1.

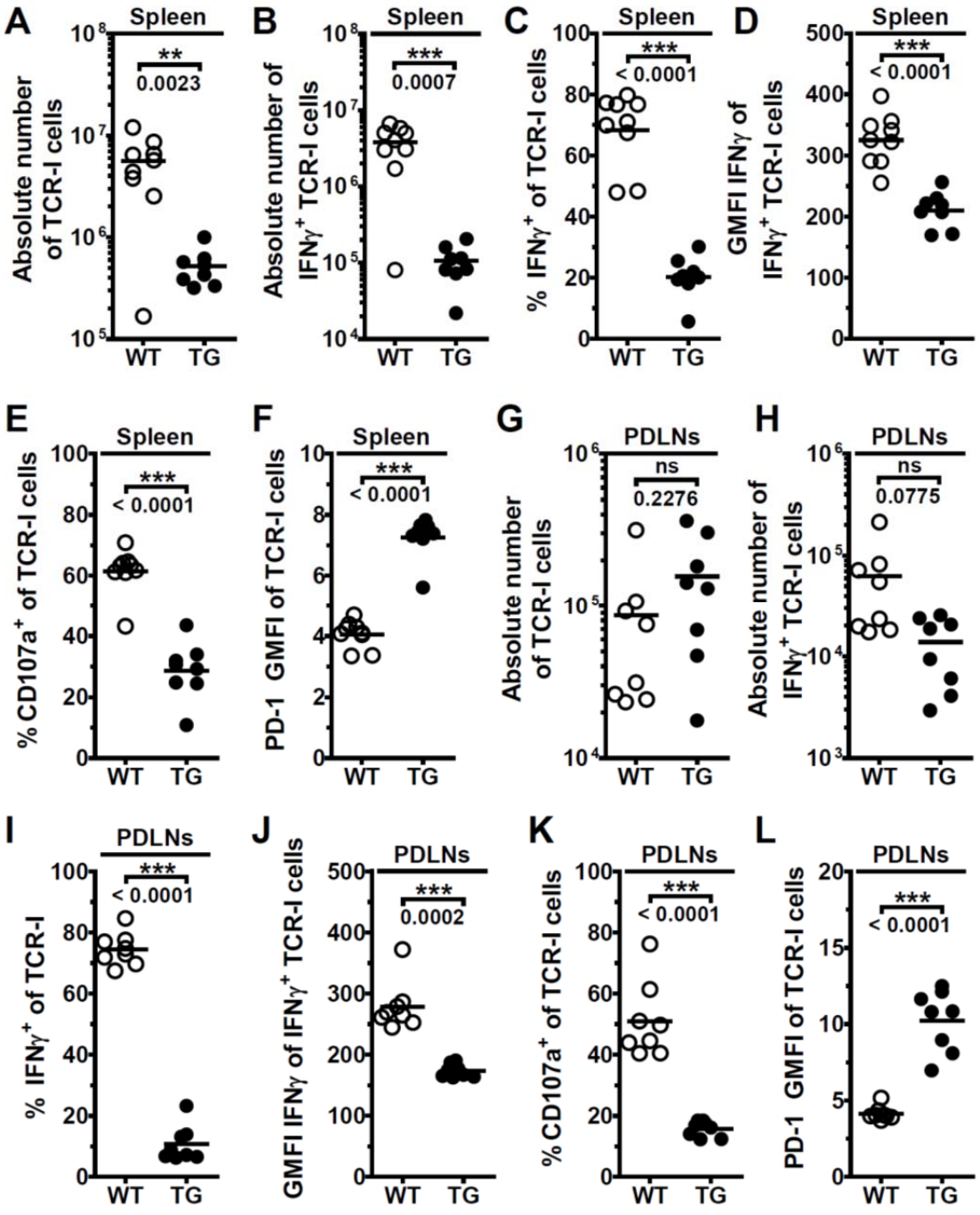


Figure S1. Tolerization of TCR-I cells in TRAMP mice

One million $CD8^+$ TCR-I cells were adoptively transferred into 14-17 weeks old male TRAMP or WT mice. Mice were infected 7 days after adoptive transfer with 2 x 10^6 PFU rVV-I. TCR-I cells were analyzed 5 days after infection. (**A, G**) Absolute number of TCR-I cells and (**B, H**) of IFN-γ producing TCR-I cells are shown for the spleen and PDLNs respectively. (**C, I**) Frequency and (**D, J**) intensity of IFN-γ production of transferred cells are illustrated. (**E, K**) Frequency of degranulating TCR-I $CD8^+$ cells in spleen and PDLNs are shown 5 days post-infection. (**F, L**) GMFI of PD-1 surface expression is displayed. Symbols represent individual mice (n=8 per group) and data from one out of three independent experiments are shown. Horizontal lines represent mean values.

Fig. S2.

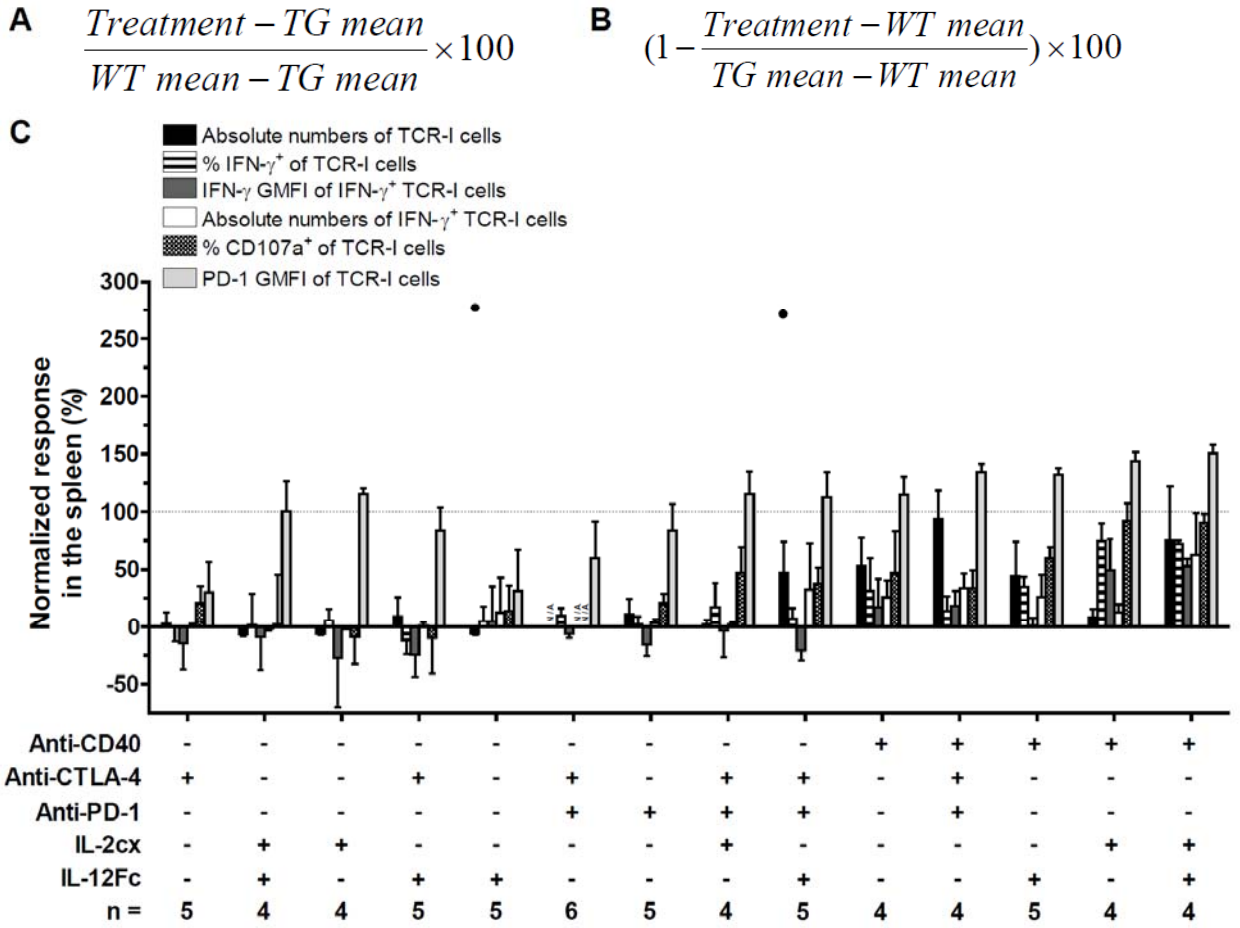


Figure S2. Preventing tolerance induction by immune intervention. One million $CD8^+$ TCR-I cells were adoptively transferred into 13-17 weeks old male TRAMP or WT mice. Mice were treated as described in Figure 5A and infected 7 days after adoptive transfer with 2 x 10^6 PFU rVV-I. (**A**) Equation used for the normalization of data to enable comparison between experiments. (**B**) Equation used for the normalization of PD-1 expression data. Normalization was performed to have WT responses averaging 100% and 0% for TG controls for all read-outs. (**C**) Normalized responses obtained from 14 different immunotherapies for 6 TCR-I read-outs in the spleen. Each bar represents a read-out as illustrated in the legend. Data are represented as mean ± SD. Two outliers identified by a significant Grubb's test were excluded from the calculations but are shown on the bar graph as circles (●).

Fig. S3.

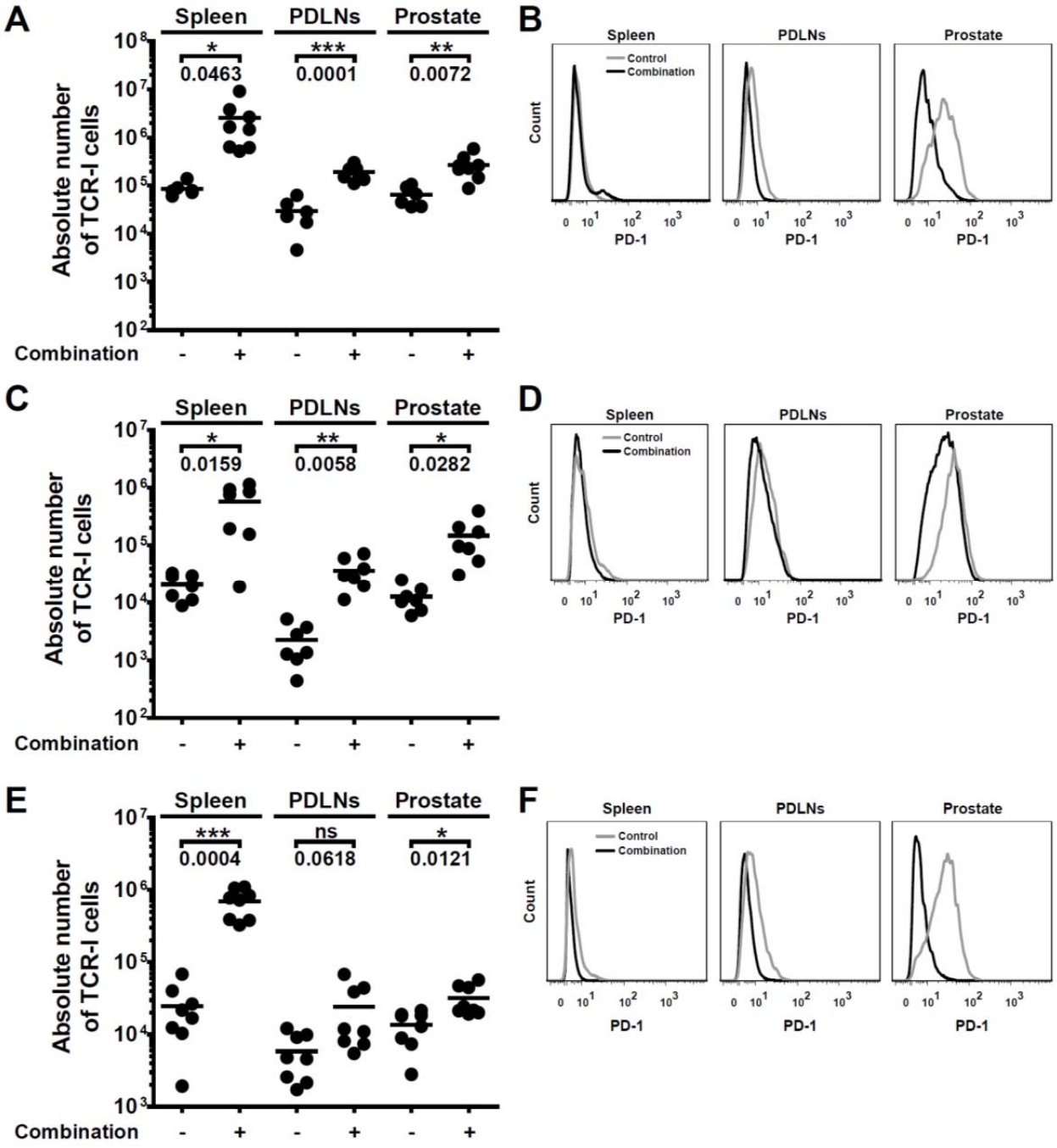


Figure S3. Administration of anti-CD40 + IL-2 complexes + IL-12Fc improves local and systemic tumor-specific immunity. One million naïve TCR-I cells were adoptively transferred into 16-18 weeks old male TRAMP mice. (**A, C, E**) Absolute numbers of TCR-I cells in the spleen, PDLNs and prostate of TRAMP mice at respective endpoints. (**B, D, F**) Surface PD-1 expression on TCR-I cells in TRAMP mice. Two representative mice are shown per panel. Panels are related to the treatment schedules in Figure 6 as follow: panels A-B (Figure 6A), panels C-D (Figure 6C) and panels E-F (Figure 6E). Symbols represent individual TRAMP mice and data from three out of seven independent experiments are shown. Horizontal lines represent mean values.

Fig. S4.

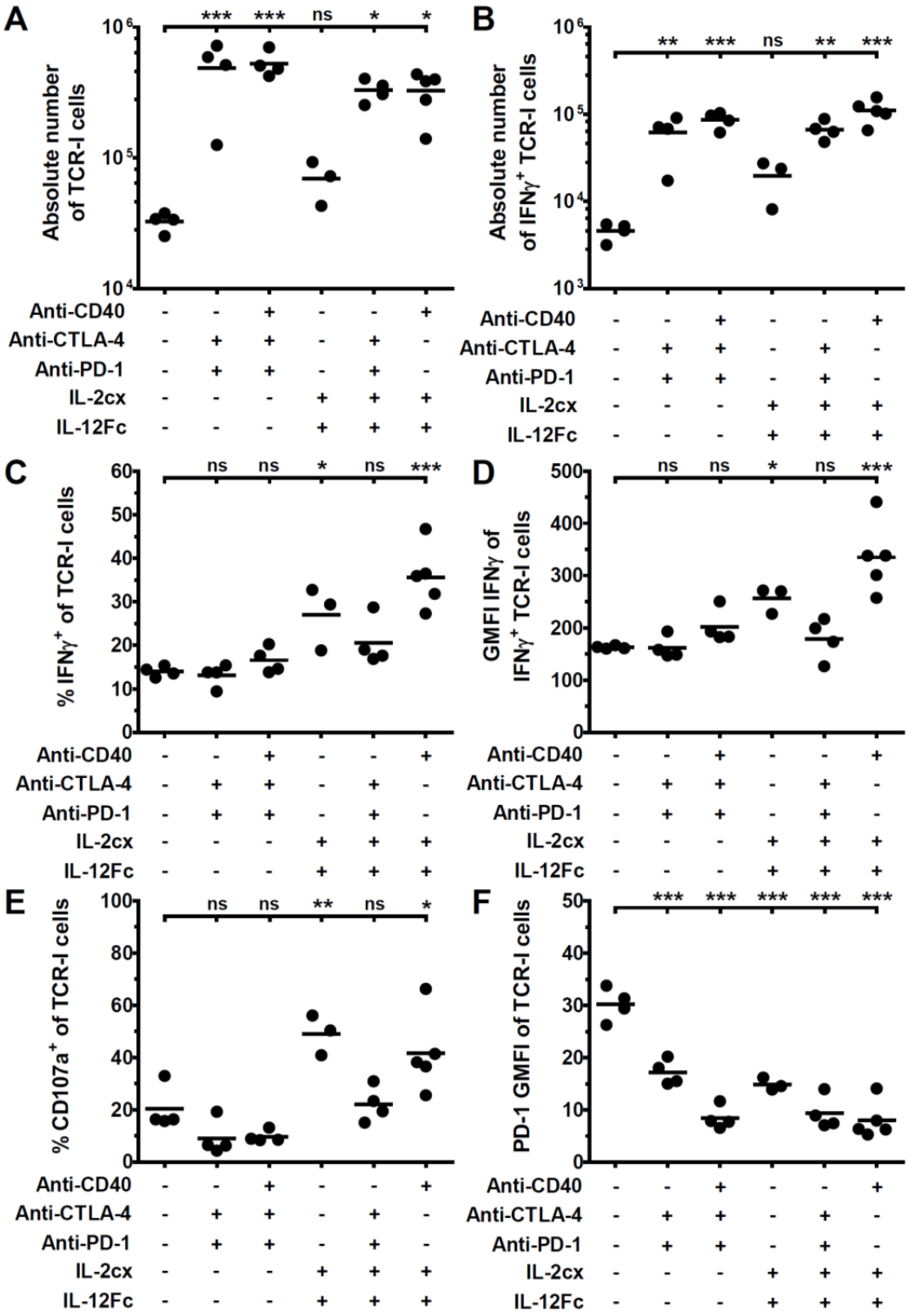


Figure S4. Immune interventions can shift the balance from tolerance into priming of tumor-specific immunity. One million naïve TCR-I cells were adoptively transferred into 14-17 weeks old male TRAMP mice. Treatment schedule was identical to Figure 6A. (**A**) Absolute number of TCR-I and (**B**) of IFN-γ^+ TCR-I cells in the prostate on day 7 after transfer. (**C**) Frequency and (**D**) intensity of IFN-γ production by TCR-I cells. (**E**) Frequency of degranulating and (**F**) PD-1 surface expression of transferred $CD8^+$ cells. Symbols represent individual mice and data from one out of two independent experiments are shown. Significant differences were determined using one-way ANOVA with Dunnett's post-test to compare all groups to the control group. Horizontal lines represent mean values.

Fig. S5.

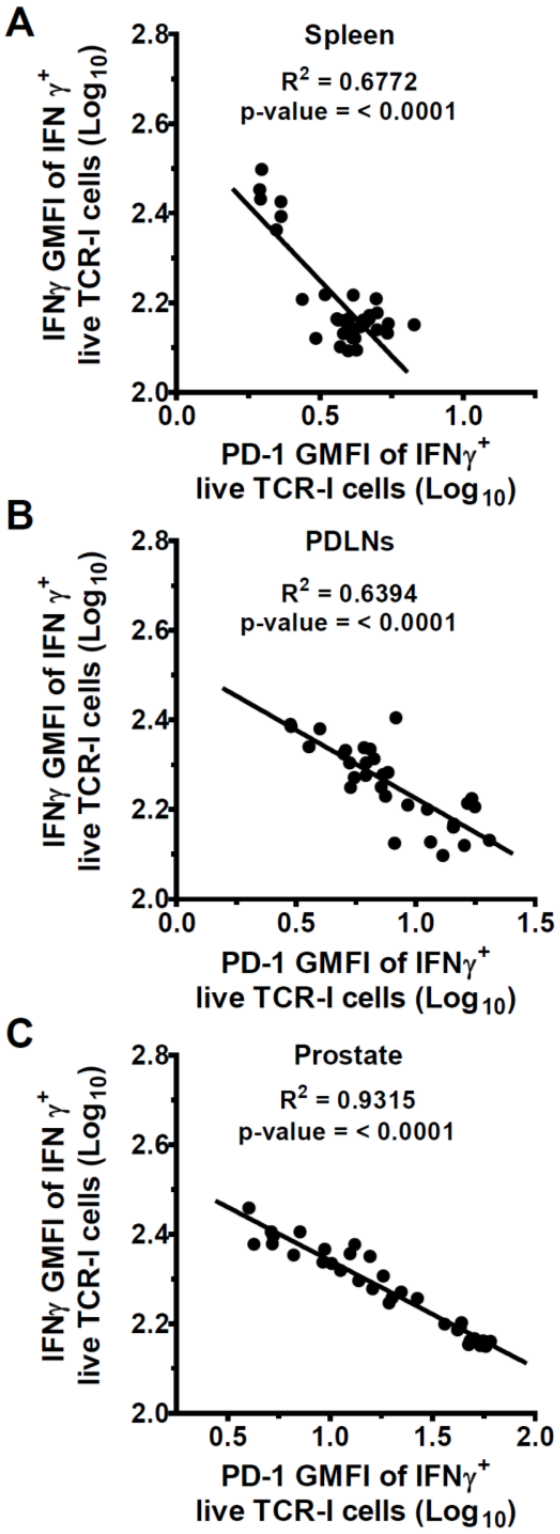


Figure S5. Inverse correlation between PD-1 surface expression level and IFN-γ production by IFN-γ$^+$ TCR-I cells. One million naïve TCR-I cells were adoptively transferred into 16-18 weeks old male TRAMP mice. Multiple groups were treated as in Figures 6A, 6D and 6G. Logarithmic transformation of PD-1 GMFI and IFN-γ GMFI gated on IFN-γ$^+$ TCR-I cells were plotted for the (**A**) spleen, (**B**) PDLNs and (**C**) prostate. Linear regressions were performed in GraphPad Prism. Samples with less than 20 IFN-γ$^+$ TCR-I cells were excluded from the correlation analysis. Mice were processed 7 or 21 days after adoptive transfer to cover a wider range of responsiveness. All samples were measured on the same day. Symbols represent individual TRAMP mice.

Fig. S6.

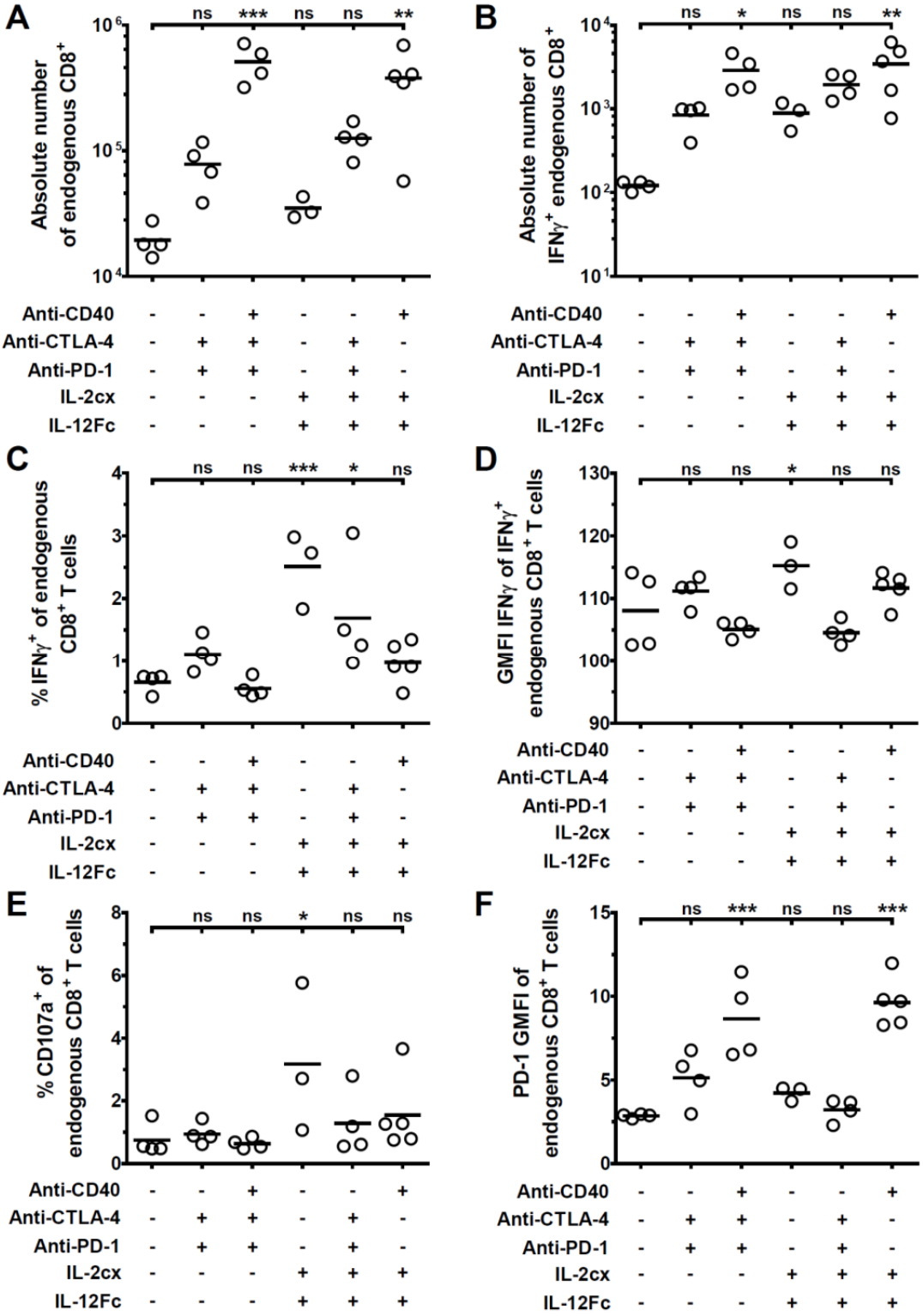


Figure S6. Immune interventions trigger tumor-specific endogenous CD8$^+$ T cell responses. Mice from Figure S4 were analyzed for endogenous CD8$^+$ T cells. Treatment schedule is depicted in Figure 6A. (**A**) Absolute number of endogenous CD8$^+$ T cells in the prostate 7 days after the start of therapy. (**B**) Absolute number of IFN-γ$^+$ endogenous CD8$^+$ T cells. (**C**) Frequency and (**D**) intensity of IFN-γ production by these cells. (**E**) Frequency of CD107a$^+$ and (**F**) PD-1 surface expression of endogenous CD8$^+$ T cells. Symbols represent individual mice and data from one out of two independent experiments are shown. Significant differences were determined using one-way ANOVA with Dunnett's post-test to compare all groups to the control group. Horizontal lines represent mean values.

Fig. S7.

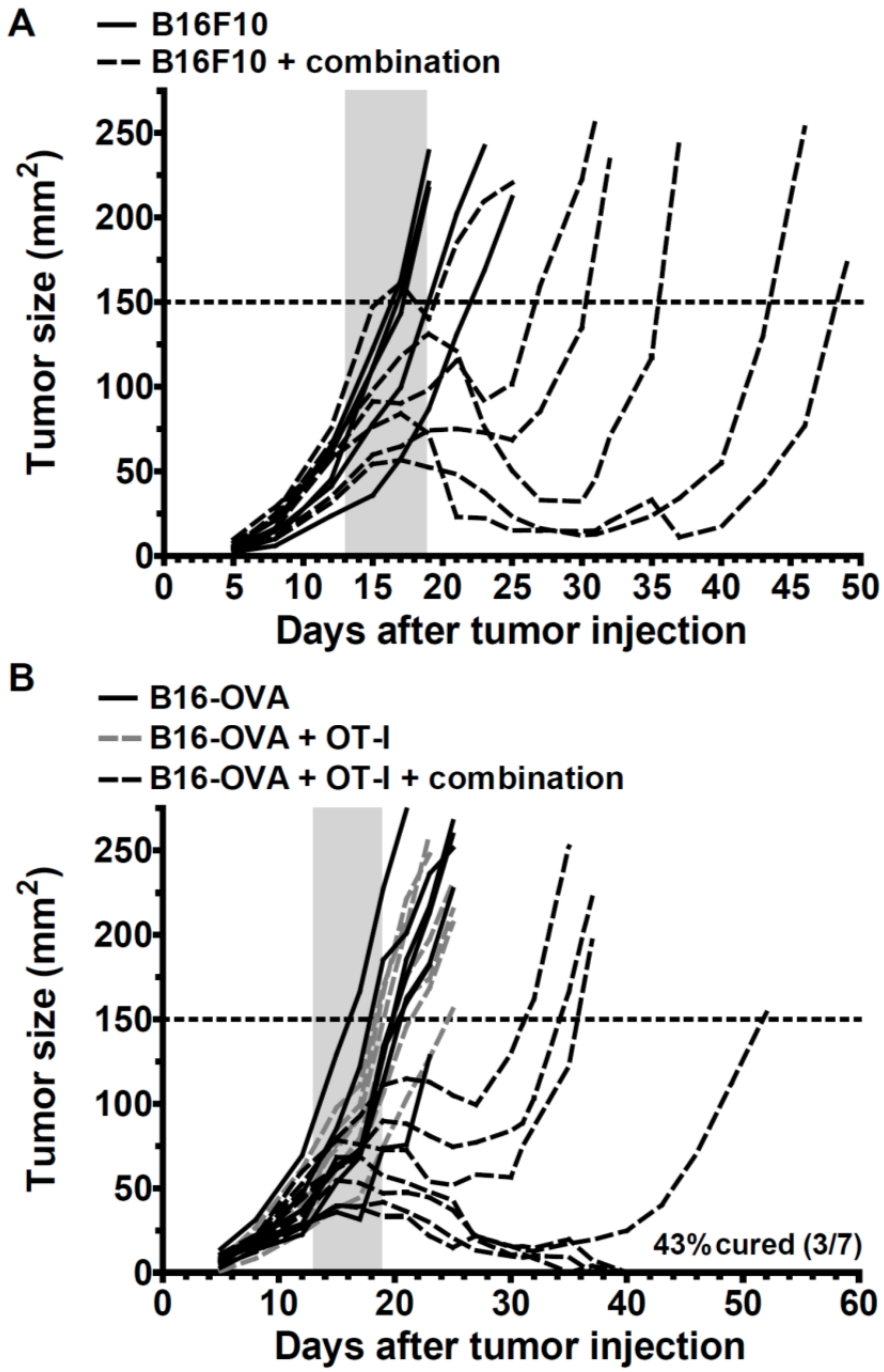


Figure S7. Tumor growth curves of subcutaneous B16 melanoma. Experimental design is described in Figure 8. (**A**) B16F10 tumor growth in C57BL/6 mice. (**B**) B16F10-OVA tumor growth in C57BL/6 mice, some of which received one million naïve OT-I $CD8^+$ cells on day 13. Gray shades on graphs mark the 7 days treatment with anti-CD40 + IL-2 complexes + IL-12Fc. Dotted line represents the cutoff considered as death event for survival analysis. Data from two out of four experiments are shown.

Fig. S8.

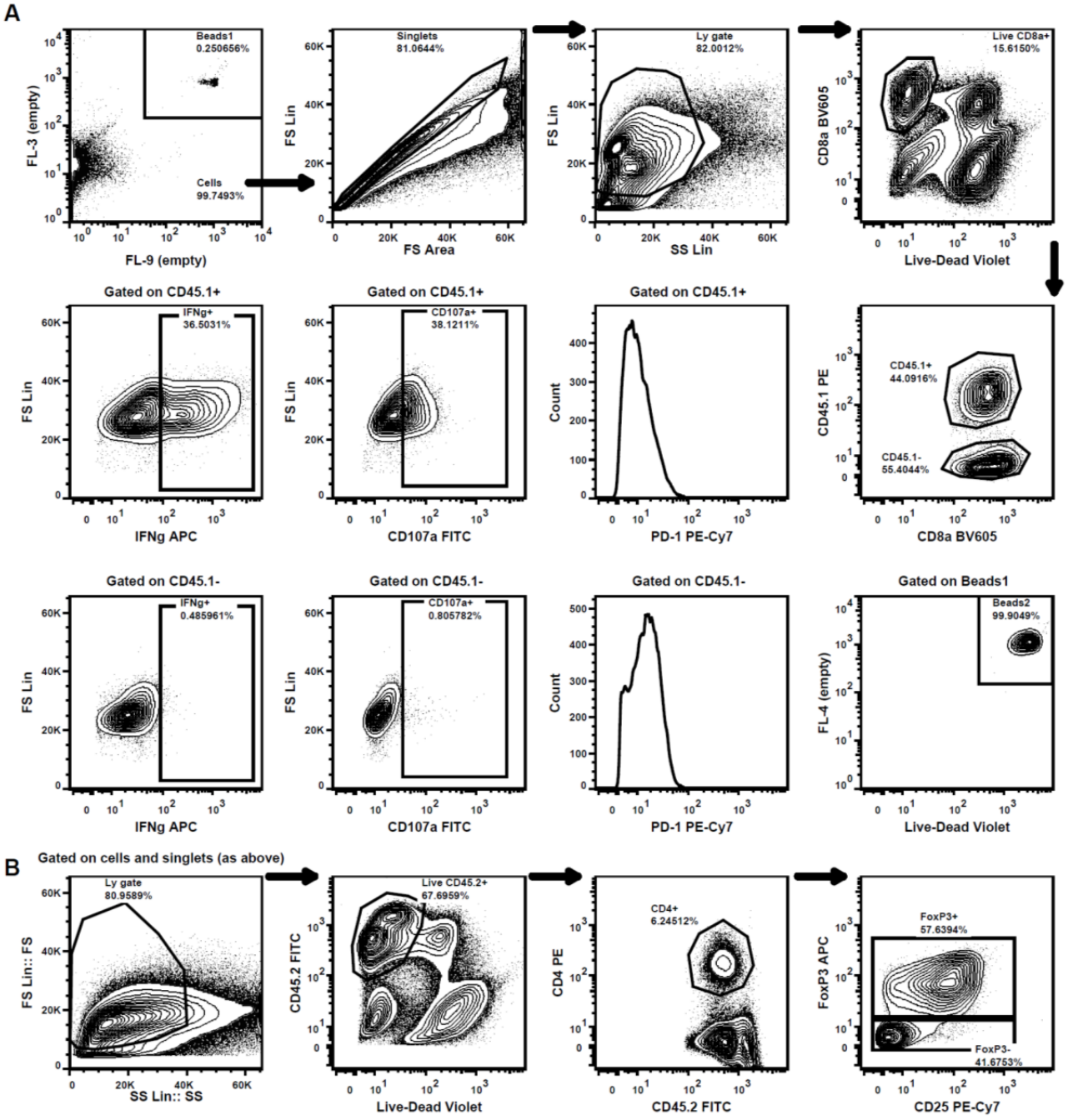


Figure S8. Gating strategy for flow cytometry analysis. Two representative examples of murine prostates are shown. (**A**) Gating strategy for $CD8^+$ endogenous and adoptively transferred TCR-I cells showing exclusion of beads, doublets and dead cells. In all experiments, counting beads were separated from cells by gating on bright events in 2 unused channels. Following gating was performed on live $CD8^+$ cells followed by CD45.1 discrimination. Representative example of IFN-γ, CD107a and PD-1 stainings are shown. (**B**) Identification of T regulatory cells was done by exclusion of beads and doublets as in (**A**) followed by gating on live $CD45.2^+$, $CD4^+$ and $FoxP3^+$ cells. CD25 was included in the staining but not considered for Treg discrimination.

6.1.8. Supplemental Tables

Table S1. Impact of various immune interventions on preventing tolerance while improving proliferation and function of TCR-I cells (Data for spleen).

Treatment	Abs. count TCR-I	% IFN-γ$^+$ of TCR-I	GMFI IFN-γ of IFN-γ$^+$ TCR-I	% CD107a$^+$ of TCR-I	GMFI PD-1 of TCR-I	Abs. count IFN-γ$^+$ TCR-I
IL-2cx + IL-12Fc + aCD40	75	72	53	90	150	63
IL-2cx + aCD40	9	74	49	92	144	13
aCTLA4 + aPD1 + aCD40	94	14	18	33	134	34
IL-12Fc + aCD40	44	35	1	60	132	26
aCD40	53	31	17	47	115	26
IL-12Fc + aPD1 + aCTLA4	47	7	-21	38	113	33
IL-2cx + aPD1 + aCTLA4	2	17	-3	47	115	2
aPD1	11	2	-15	20	84	3
aPD1 + aCTLA4	N/A	10	-6	N/A	60	N/A
IL-12Fc + aCTLA4	9	-12	-24	-10	84	1
IL-2cx	-6	6	-27	-9	116	-2
IL2cx + IL12Fc	-7	1	-9	2	100	-2
IL12Fc	-6	5	5	14	31	12
aCTLA4	3	0	-15	21	30	1

Tables S1, S2 and S3, Related to Figures 5 and S2. Impact of various immune interventions on preventing tolerance while improving proliferation and function of TCR-I cells. Exhaustive compilation of all normalized data (see formulas in Figure S2) obtained with spleen (Table S1) and PDLNs (Table S2) of treated mice. Treatments are ranked by global average of all spleen and PDLNs values and the 95% confidence interval for this average is shown (Table S3). The 3 best responses per read-out per organ are highlighted in gray. Absolute counts of TCR-I cells and counts of IFN-γ$^+$ TCR-I in PDLNs were excluded from calculations since no significant differences were observed between WT and TG control mice (Figures S1G and S1H). Two outliers identified by a significant Grubb's test shown in Figure S2 were also excluded from the calculations.

Table S2. Impact of various immune interventions on preventing tolerance while improving proliferation and function of TCR-I cells (Data for PDLNs).

Treatment	% IFN-γ^+ of TCR-I	GMFI IFN-γ of IFN-γ^+ TCR-I	% CD107a$^+$ of TCR-I	GMFI PD-1 of TCR-I
IL-2cx + IL-12Fc + aCD40	94	121	86	115
IL-2cx + aCD40	56	88	43	109
aCTLA4 + aPD1 + aCD40	40	16	84	94
IL-12Fc + aCD40	55	48	67	82
aCD40	29	-5	54	87
IL-12Fc + aPD1 + aCTLA4	40	24	52	78
IL-2cx + aPD1 + aCTLA4	2	-22	7	86
aPD1	17	15	23	65
aPD1 + aCTLA4	14	9	N/A	44
IL-12Fc + aCTLA4	18	-4	24	49
IL-2cx	-2	-25	-28	95
IL2cx + IL12Fc	7	-30	-4	47
IL12Fc	18	-26	17	-13
aCTLA4	-8	-30	-11	6

Table S3. Impact of various immune interventions on preventing tolerance while improving proliferation and function of TCR-I cells (Average of spleen and PDLNs data).

		95% Confidence Interval	
Treatment	**Global Average**	**Lower Limit**	**Higher Limit**
IL-2cx + IL-12Fc + aCD40	92	81	103
IL-2cx + aCD40	68	54	82
aCTLA4 + aPD1 + aCD40	56	43	70
IL-12Fc + aCD40	55	44	65
aCD40	45	33	57
IL-12Fc + aPD1 + aCTLA4	41	29	53
IL-2cx + aPD1 + aCTLA4	25	11	40
aPD1	23	13	32
aPD1 + aCTLA4	22	12	31
IL-12Fc + aCTLA4	13	3	24
IL-2cx	12	-5	28
IL2cx + IL12Fc	11	-3	24
IL12Fc	6	-3	14
aCTLA4	0	-7	6

6.2. Generation of a new transgenic mouse model (ARTURO)

6.2.1. Introduction

Simian virus 40 expresses the dominant acting oncoprotein large T antigen (SV40LT) that elicits cellular transformation mostly by associating with key cellular proteins and altering the signaling pathways of the tumor suppressors p53 and Rb-family (*102*). Several reasons argue for the use of SV40LT-based models for the investigation of the interplay between tumors and tumor-specific immunity: (i) SV40LT expression results in reliable tumor induction, independent of the tissue (*103-107*), (ii) in contrast with transplanted tumor cell lines, SV40LT-tumors are an accepted model for spontaneous tumors with a relatively slow genesis and the presence of a relevant tumor microenvironment (*93, 108-111*), (iii) several H-2^b-restricted T cell epitopes have been identified in SV40LT, allowing precise dissection of SV40LT-specific immunity during tumorigenesis (*112, 113*).

To generate a new transgenic mouse model that allows the induction of tumors in a tissue of interest, we targeted a conditional transgene cassette to the well-characterized Rosa26 euchromatic locus by homologous recombination in mouse embryonic stem cells (mES cells). Targeting the transgene to the Rosa26 locus results in reliable transgene expression and minimizes the risk of an unwanted phenotype due to random integration as observed after a conventional transgenic approach (*114*). The targeting construct was designed to achieve homologous recombination at the target locus to replace native Rosa26 locus sequences with a large transgene cassette as depicted in Figure 10. The 5' short and 3' long homology arms (SA and LA) in bright pink consist of several kilobases identical to the C57BL/6 Rosa26 locus enabling the cell's DNA repair machinery to substitute wild-type DNA for the transgenic cassette through homologous recombination (*115*).

The targeting construct contained a loxP-flanked stop cassette downstream of a ubiquitous promoter (CAG). The CAG promoter consists of a CMV enhancer sequence, the chicken β-actin promoter and the splice acceptor of the rabbit β-globin gene, which allows strong gene expression in mammalian cells (*116*). The floxed-stop cassette contains sequences to block ubiquitous expression of the transgenes while also including the sequence coding for neomycin to facilitate selection of targeted mES clones (*115, 117*).

The vector was modified to introduce the transgenes downstream of the stop cassette (Figure 10) to enable conditional expression in double-transgenic offspring when crossing ARTURO mice with tissue-specific Cre^{ERT}-expressing mice (*115, 117-119*). Cre^{ERT} is a fusion protein between the Cre recombinase that mediates floxed sequence excision and the mutated hormone-binding domain of the human estrogen receptor (ERT) (*120*). In absence of tamoxifen, the synthetic ligand for ERT, the Cre recombinase is associated with heat shock proteins, which inhibit translocation of Cre into

the nucleus. After association of ERT with its ligand, Cre can translocate into the nucleus and remove loxP-flanked nucleotides.

The expression cassette of our targeting construct contains the SV40LT to drive carcinogenesis, the firefly luciferase to enable non-invasive monitoring of arising tumors and metastases by luminescence with an IVIS imaging system (*121, 122*) and enhanced green fluorescent protein (eGFP) to enable identification of tumor cells by flow cytometry. Thus, double-transgenic ARTURO x TS-Cre^{ERT} mice should only express the transgenes in a tissue of choice after injection of tamoxifen, which will result in tumor development of this particular tissue. By comparing fundamentally different target tissues, we aimed to find mechanisms that preclude immune-mediated tumor control that are active in every malignancy and some that are organ-specific. In fact, there is growing evidence that the complexity of the immune infiltrate is influenced by the tissue where the tumor originates and the stage of malignancy, as observed by the analysis of distinct tumors by flow cytometry (*123*). These variations suggest that the tumor milieu may differ significantly between primary and metastatic lesions. Since the immune networking in tumors is of high functional and clinical relevance (*35, 36*), it is important to consider these qualitative and quantitative differences in the tumor microenvironment for immune-based therapies. For example, immunotherapies that rely on antibody-dependent cell-mediated cytotoxicity (ADCC) may require a subset of cells with a specific FcγR at the tumor site for their efficacy (*124-127*).

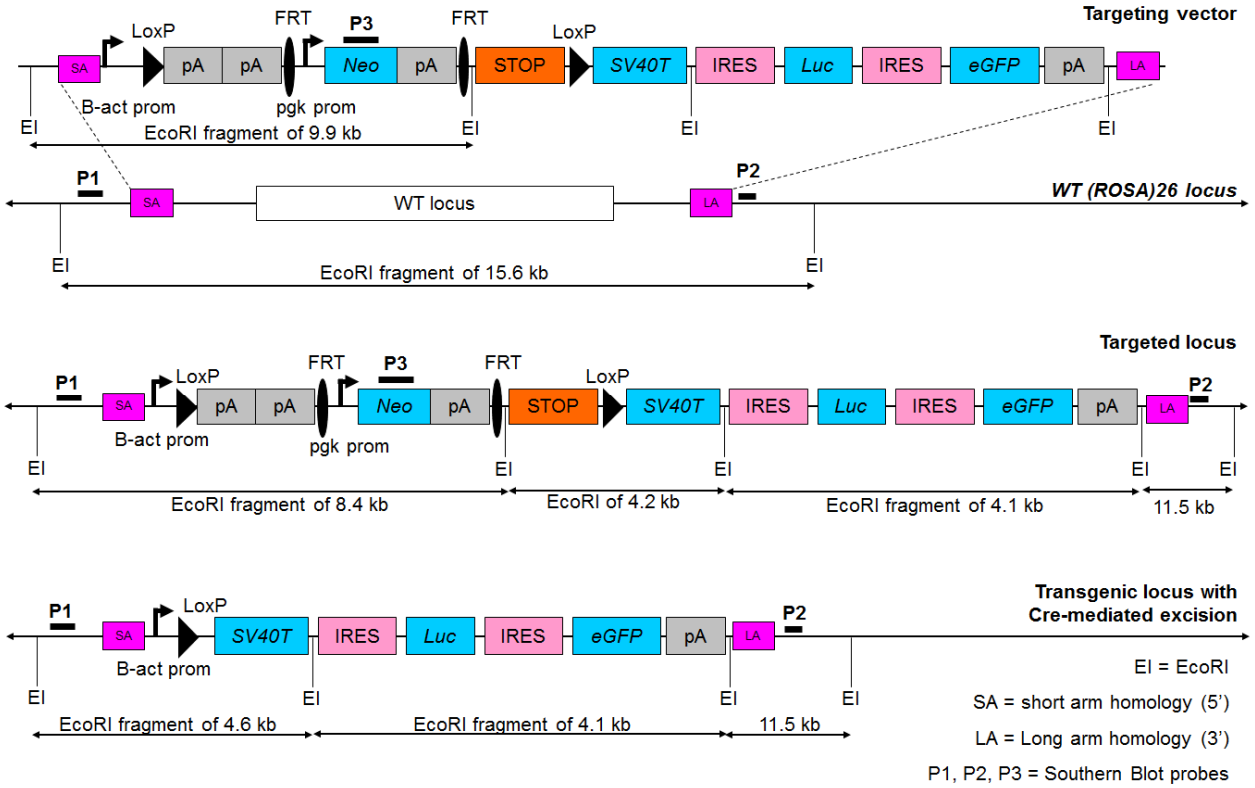


Figure 10. Schematic strategy for targeting and screening of mouse ES cells.

Figure 11. Schematic description for the induction of tissue-specific tumorigenesis in double-transgenic mice.

6.2.2. Material and methods

6.2.2.1. Cloning of targeting construct

A targeting construct (BBV43GFP-GatewayMod) containing homology arms for the Rosa26-locus (SA and LA), a loxP-flanked stop cassette downstream of the CAG promoter, a polylinker including gateway recombination cloning sites (attR1 and attR2) and the IRES-eGFP-pA cassette was kindly provided to us by Sergio Haller (Institute for Experimental Immunology, University of Zurich). For the cloning, SV40LT was digested (EcoR1 + Xba1) and gel-purified from the template vector pBS-Tag(wt). The purified fragment was ligated upstream of the IRES-Luc cassette in the linearized (Nhe1 + EcoR1) pGL3-Basic luciferase reporter vector. Subsequently, the SV40LT-IRES-Luc cassette was PCR amplified with the following primers: 5'-TTT TGT CGA CGC CGC CAC CAT GGA TAA AGT TTT AAA CAG-3' and 5'-TTT TGC GGC CGC TTA CAC GGC GAT CTT TCC-3'. The purified PCR product was cloned (Sal1 + Not1) into the gateway pENTR11 dual selection entry vector. Gateway recombination (Gateway LR Clonase, Life Technologies) was performed between the vectors pENTR11-SV40LT-IRES-Luc (entry clone with attL sites) and BBV43GFP-GatewayMod (destination vector with attR sites) according to manufacturer's protocol. The recombined DNA mixture was electroporated into SURE electroporation-competent *E. coli* cells (Stratagene) to avoid rearrangement and deletion of non-standard secondary and tertiary structures that occur frequently in eukaryotic DNA. Although SURE cells were used, abnormal rearrangements could only be prevented by incubating SURE cells at 25°C instead of 37°C. All enzymes were purchased from New England Biolabs unless otherwise stated.

6.2.2.2. Generation and identification of targeted ES cell

Amplification, linearization (SgrD1) and purification of the targeting construct was performed and shipped to the Transgenics & Gene Targeting Facility at the Alexander Fleming

Biomedical Sciences Research Center in Vari, Greece. The targeting service was done by electroporating the linearized construct into Bruce-4 murine embryonic stem cells (*128*). Neomycin selection was used to isolate transgenic ES cell clones. Cells were expanded in 96-well plates and the frozen pellets were transferred back in Zurich for the identification of clones with single and proper integration at the Rosa26 locus. Southern blotting was carried like previously published (*115, 119*) using 3 different radio-labeled probes (P1, P2 and P3 in Figure 10). Briefly, genomic DNA was extracted, digested with EcoR1, migrated on 0.7% agarose gel and followed by alkaline transfer onto Zeta-Probe cationized nylon membrane (Bio-Rad). Radioactive labeling of probes was performed with [α-^{32}P]-dCTP using the Takara Ladderman labeling kit. Overnight hybridization of membranes with the probes was done at 65ºC in a rotating incubator. After multiple washing steps, binding of probes was detected with a PhosPhor screen (GE Healthcare) and digitized using a phosphor imaging system.

6.2.2.3. Generation of transgenic line

Following the identification of multiple targeted ES cell clones, 5 clones were expanded and karyotyped by the Transgenics & Gene Targeting Facility in Greece. Selected clones were used for blastocysts injections and transferred into C57xCBA foster mice to generate chimeric mice (see Figure 14). Chimeras were bred to C57BL/6J-Tyrc-2J (albino) mice and the progeny was genotyped for the presence of the transgene.

6.2.2.4. Genotyping of progeny

Genotyping of pups was performed by multiple PCRs enabling identification of the targeted-locus (Rosa26 PCRs) or simply the presence of transgene (SV40LT). Reactions with FIREPol DNA Polymerase (Solis BioDyne) were performed according to manufacturer's instructions using the primers in Table 2. Genomic DNA was extracted from mouse tissues with the rapid NaOH lysis as reported previously (*129*).

Primer	Sequence (5'→3')	Binding	Pairing	Amplicon (bp)
AB102	TTT GCC AGG TGG GTT AAA GGA GCA	SV40LT	AB103	See below
AB103	CAG CAG AGC CTG TAG AAC CAA ACA	SV40LT	AB102	WT = N/A TG = 400
AB106	AAA GTC GCT CTG AGT TGT TAT	Rosa26 (5' SA)	AB107 and AB108	See below
AB107	GGA GCG GGA GAA ATG GAT ATG	Rosa26 (3' LA)	AB106	WT = 600 TG = N/A
AB108	CAT CAA GGA AAC CCT GGA CTA CTG	CAG promoter	AB106	WT = N/A TG = 241

Table 2. Primers for PCR genotyping of chimeras and progeny.

6.2.3. Results

6.2.3.1. Targeting of murine embryonic stem cells

Following the cloning and sequencing of the targeting construct, the purified vector was linearized and sent to the Transgenics & Gene Targeting Facility at the Alexander Fleming Biomedical Sciences Research Center in Vari, Greece. Targeting was performed in Bruce-4 ES cells, one of the most widely used C57BL/6 ES cell line for their high targeting efficiency and proven germ line transmission (Figure 12) (*128*). The targeting construct was transferred by electroporation and 384 neomycin-resistant ES cell clones were isolated.

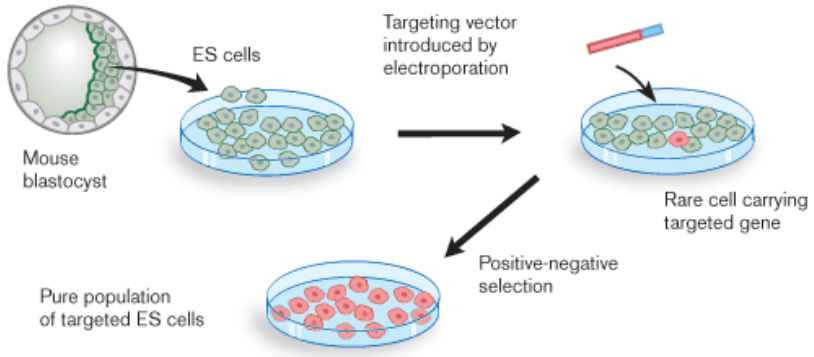


Figure 12. Gene targeting of embryonic stem cells. Figure from Annika Röhl, www.partners.org

Identification of clones with correct integration was determined by southern blotting of EcoRI-digested genomic DNA (gDNA). Three probes were used, P1 and P2 to assess integrity at both ends of the insertion and P3, an internal probe, to confirm single integration event (Figure 10). Targeting efficiency was 14% with a total of 54 clones with expected southern blot patterns (data not shown). Five targeted clones (A5, B8, D8, F12 and G9) and two neomycin-resistant non-targeted controls (C2 and C4) were selected for expansion and confirmation of southern blots (Figure 13). Random integration for C2 and C4 is illustrated by the absence of transgenic (TG) bands for P1 and P2 while P3 detects a fragment of inappropriate size.

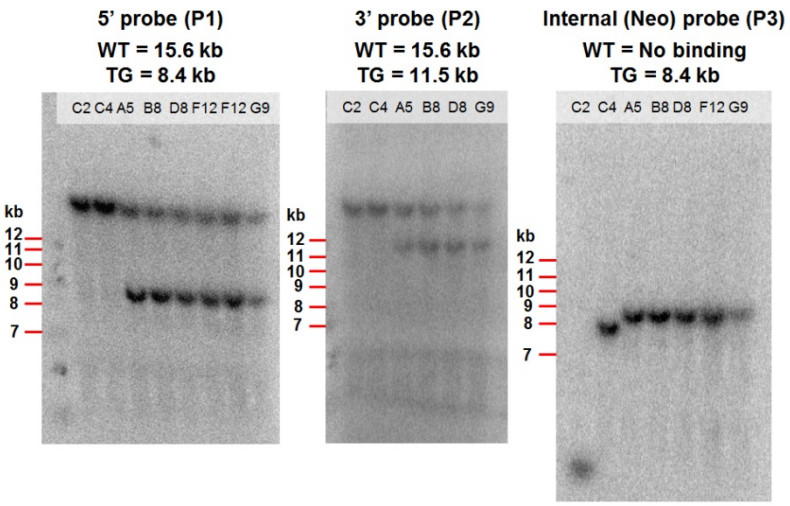


Figure 13. Southern blot confirmation of proper integration of the transgene into the Rosa26 locus.

6.2.3.2. Generation and breeding of chimeras

Subsequently, ES cell clones were injected into albino blastocysts and transferred to foster mice (Figure 14). Albino blastocysts were used to allow visual discrimination of chimeric mice since transgenic Bruce-4 ES cells lead to development of black and white fur while non-chimeric mice remain albino. The 216 blastocysts injections at the Transgenics & Gene Targeting Facility in Greece generated a total of 36 pups, of which 17% (6 / 36) were chimeric. One chimera died unexpectedly before sexual maturity. Figure 15 shows the five other chimeras and some albino pups. Breedings between albino mice and these five chimeras were performed and are summarized in Table 3.

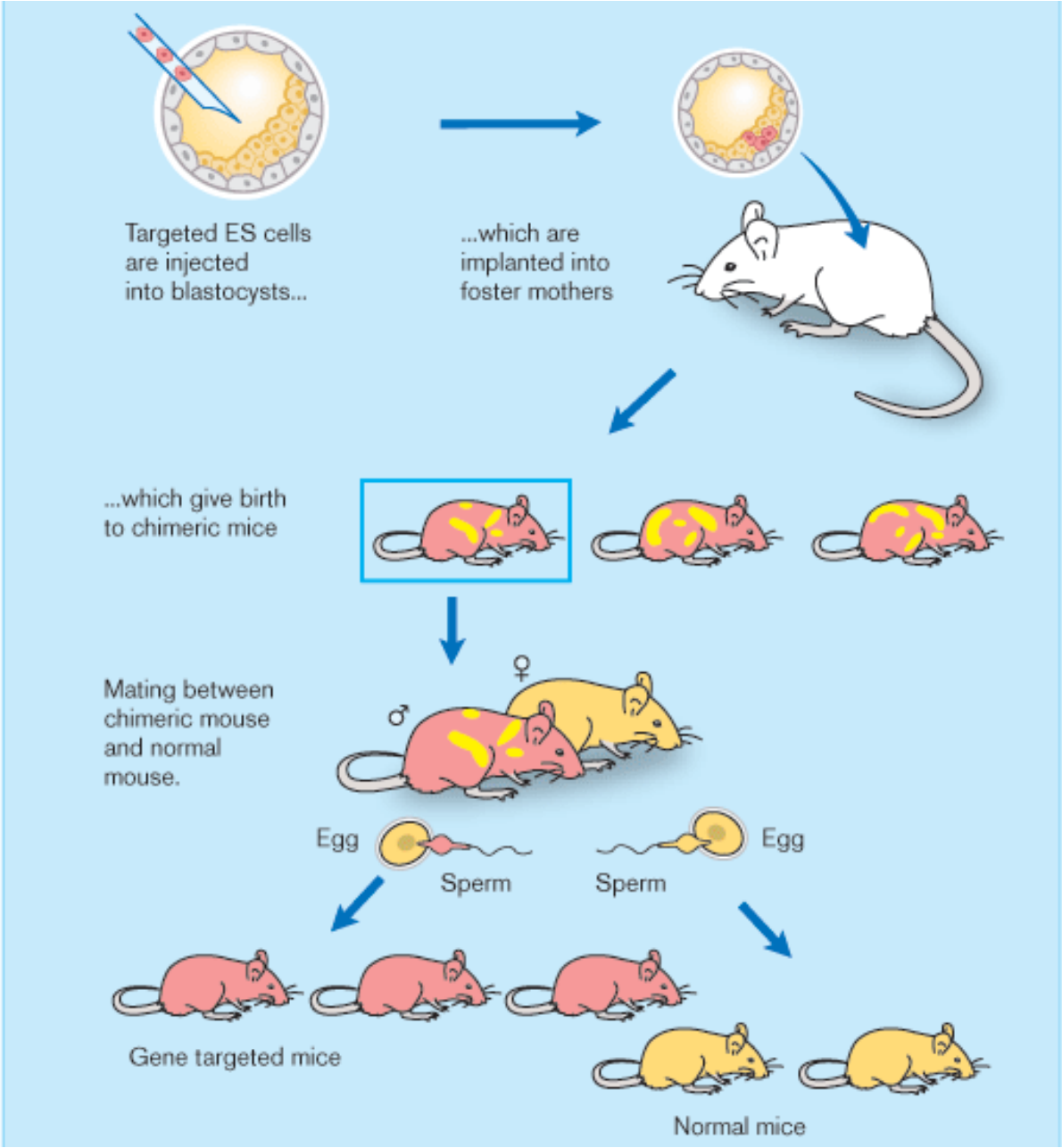


Figure 14. Generation of gene targeted mice. Figure from Annika Röhl, www.partners.org

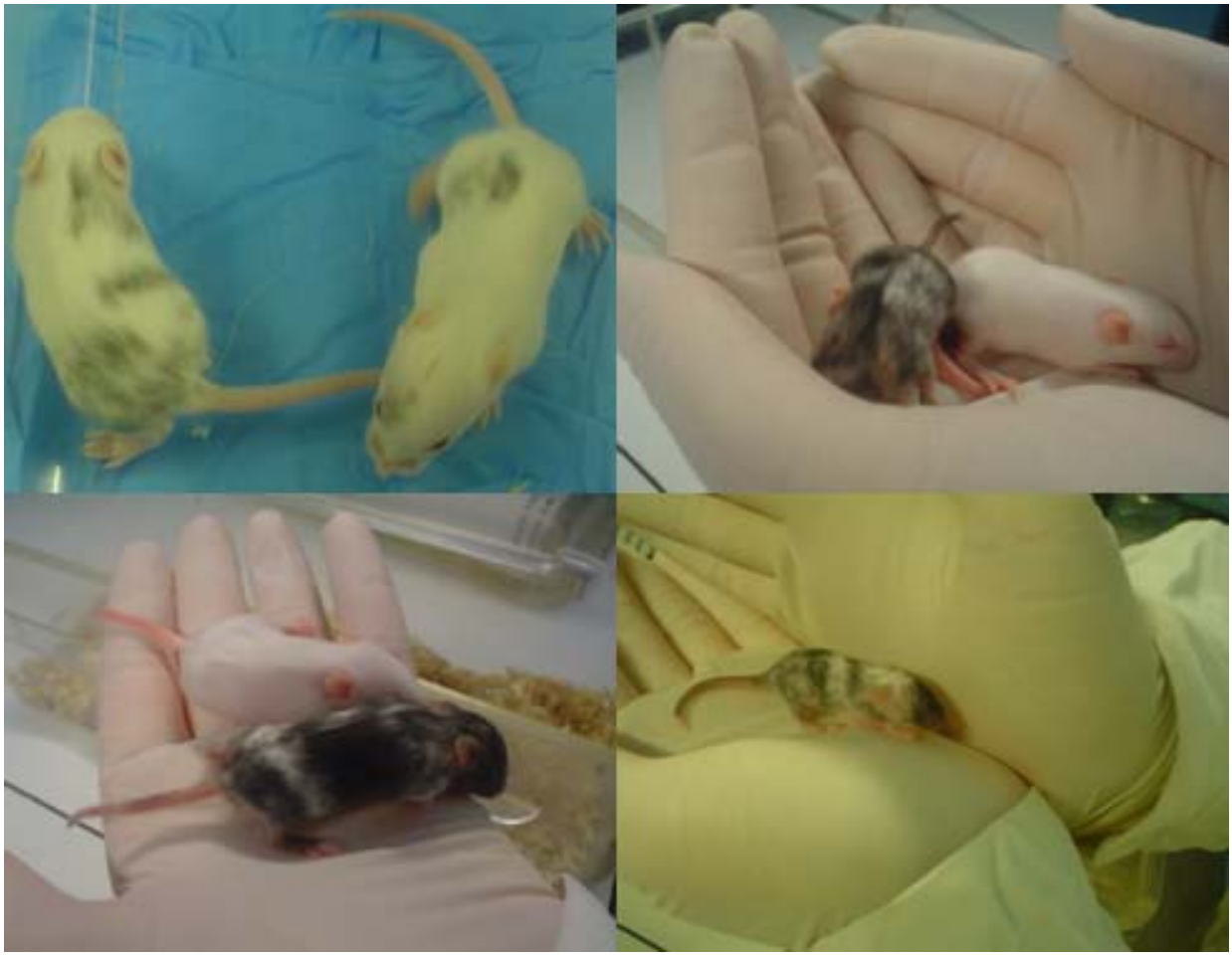
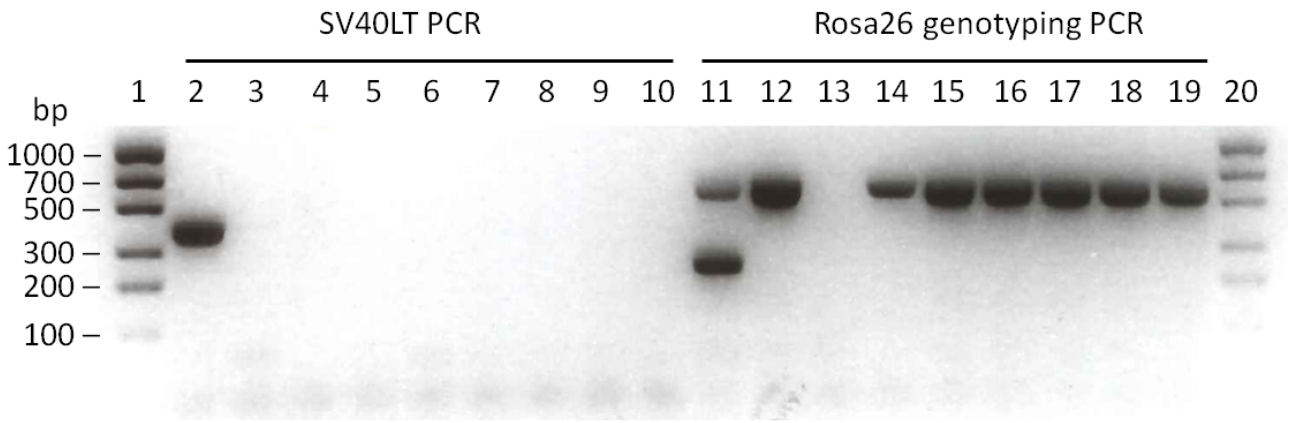


Figure 15. Chimeras obtained after injection of targeted ES cells into blastocysts.

	Chimera 1	**Chimera 2**	**Chimera 3**	**Chimera 4**	**Chimera 5**	**Chimera 6**
Sex	Male	Female	Female	Male	Male	Male
Mouse ID	B-D8-1M	B-D8-2F	B-D8-3F	B-A5-4M	B-A5-5M	B-G9-6M
Litters produced	2	2	0	3	3	3
Total pups	14	10	0	62		
# of black pups	0	0	0	10		
# of transgenic black pups	0	0	0	0		

Table 3. Summary of breedings of chimeric mice with albino mice.

Based on the results of the breedings and genotyping shown in Table 3 and Figure 16, no transgenic pups in the chimeras' progeny could be identified. Chimeras were then sacrificed and biopsies were taken from a chimera producing black pups. PCR genotyping was performed on this chimera's samples to confirm the presence of the transgene in the reproductive tract. Surprisingly, genotyping revealed the lack of the transgene in all samples tested (Figure 17).

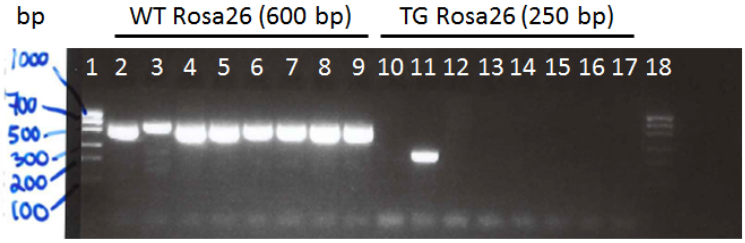


Lanes 1,20 = MassRuler Express LR DNA ladder
Lane 2, 11 = Positive control (Targeted ES cells)
Lane 3, 12 = Negative control (C57BL/6 WT gDNA)
Lane 4, 13 = Negative control (No template)
Lanes 5-10 and 14-19 = Chimera's progeny (5 pups)

SV40LT PCR expected bands:
WT = no bands, TG = 400 bp

Rosa26 PCR expected bands:
WT = 600 bp, TG = 250 bp

Figure 16. PCR genotyping of black progeny.



Lanes 1,18 = MassRuler Express LR DNA ladder
Lane 2, 10 = Negative control (C57BL/6 WT gDNA)
Lane 3, 11 = Positive control (Targeted ES cells)
Lane 4, 12 = gDNA from chimera's spleen
Lane 5, 13 = gDNA from chimera's testis
Lane 6, 14 = gDNA from chimera's sperm
Lane 7, 15 = gDNA from chimera's liver
Lane 8, 16 = gDNA from chimera's kidney
Lane 9, 17 = gDNA from chimera's intestine

Figure 17. PCR genotyping of chimera 5.

6.2.4. Discussion and outlook

Germline transmission of the transgene was not achieved so far. The southern blots proved that the ES cells were successfully targeted, but the fact that all tissues from chimera 5 were negative for the transgene may explain the lack of germline transmission.

Currently, we don't know why the tissues from chimera 5 were transgene-negative. One possibility might be that the ES cell clone used for injection did not represent a pure population and some aberrant cells overgrew the transgenic ES cells during *in vitro* expansion or following injection. Toxicity of the transgene can't be excluded, but the conditional nature of the construct should in theory protect from toxicity in absence of Cre recombination. In fact, around half of the black pups died early after birth, which may support developmental toxicity of the transgene. However, considering the absence of the transgene in the chimera and the fact that albino progeny also had a high mortality rate precludes any conclusions.

Another possibility that could explain the absence of the transgene in chimera 5 is transgene instability. The integrated portion of the construct (15 kb) contained multiple repeated regions that

may increase genomic instability. It is conceivable that the absence of neomycin selection increases the possibility of disruption at the Rosa26 locus. Sequencing of the Rosa26 locus of the chimeras or southern blotting could provide data to better understand if this is happening or not. Finally, a human error leading to the use of the wrong clone for injection could explain transgene-free chimeras.

Ultimately, bringing this project to completion requires the generation of more chimeras, including southern blot reconfirmations of the targeted clones to use for blastocysts injections and successful outcome is not guaranteed.

7. Discussion

Immunotherapy has come a long way since the first reports of a relationship between infection and cancer regression during the 18 and 19th centuries (*130, 131*). Nowadays, combined cancer death rate has been steadily declining for over 2 decades (*94*) and this success can be partially explained by the emergence of new treatments, including modern immunotherapies. Currently approved immunotherapies include: cytokines (IL-2, IFN-α) (*132, 133*), checkpoint blockade antibodies (anti-CTLA-4, anti-PD-1) (*134-136*), cancer vaccines (Sipuleucel-T) (*79*), pattern recognition receptors agonists (imiquimod, BCG) (*137, 138*) and a plethora of tumor targeting antibodies including Bevacizumab and Rituximab to name a few (*139, 140*). In addition, there are several other approaches indicating feasibility and efficacy in humans that have not reached approval yet. These promising treatments undergoing clinical trials include several combinations of checkpoint blockade with radio- or chemotherapy (*141*), personalized cancer vaccines (*142, 143*) and adoptive T cell transfers (*80*).

Fighting cancer with immunotherapy has multiple advantages over other treatments. First, immunotherapies are usually relatively well tolerated and are less toxic than aggressive chemotherapeutic regimens, for example in patients with decreased renal function (*144*). Second, immune modulation can result in systemic tumor-specific responses and therefore can prevent or control metastasis (*70, 75, 145, 146*). Third, such treatment can induce sustained tumor-specific immunity (*134, 147-150*). Fourth, the immune system can eliminate quiescent as well as dividing tumor cells while conventional treatments like radio- and chemotherapy preferentially attack dividing cells. Last but not least, immunotherapy shows clinical efficacy in cancer patients who are not responding to any other treatments (*134, 151, 152*).

In order to achieve sustained clinical efficacy with cancer immunotherapy, it is necessary to overcome T cell dysfunction, which is a consequence of suboptimal priming and the immunosuppressive tumor microenvironment. Here, we described a powerful experimental strategy for the *in vivo* evaluation of interventions with respect to the improvement of tumor-specific immunity and prevention of tolerance induction in $CD8^+$ T cells using a clinically relevant, autochthonous mouse model of prostate cancer. We identified the combination of anti-CD40 + IL-2cx + IL-12Fc as uniquely efficacious in inducing protective, long-lasting tumor-specific immunity of adoptively transferred tumor-specific $CD8^+$ T cells. This immune intervention had clinical efficacy in late stage preclinical cancer models including curative potential.

Our study highlights the qualitative and quantitative importance of signals 2 and 3 in priming protective T cell immunity. Our findings are in line with recent evidence showing that the sum of intracellular inputs resulting from antigen affinity, costimulation and cytokines dictates the

extent of T cell expansion (*59*). In this elegant study, Marchingo et al. quantified the relative contribution of different pathways to T cell division destiny. Their data showed that different costimulatory molecules (CD28 and CD27) and cytokines (IL-2, IL-12) sum up linearly to impact on the number of mitotic cycles a T cell undergoes after activation. Moreover, this effect can be titrated based on the concentration of cytokines used. This work identified a quantitative basis for the current three-signal model of T cell activation.

Although the combination of anti-CD40 + IL-2cx + IL-12Fc outperformed all other combinations we tested, it is possible that combinations of different costimulatory and cytokine signals represent alternative means to generate T cell responses of similar or even higher magnitude. Indeed, although CD27, CD28, IL-2 and IL-12 represent well-known proteins involved in signals 2 and 3, many more molecules contribute to the equation, including but not limited to those illustrated in Figure 8 of the introduction (*60*). This may explain in part why the combination of anti-CD40 + IL-2cx + IL-12Fc was so efficient compared to the other combinations we tried. Indeed, anti-CD40 can strongly improve the quality of priming by its capacity to license antigen-presenting cells (*153-155*). Our results showed that anti-CD40 + IL-2cx + IL-12Fc supported differentiation of adoptively transferred $CD8^+$ T cells into protective effectors. Moreover, this therapy also generated a memory pool detectable 6 months after treatment, which provided systemic, long-lasting cancer surveillance, probably contributing to the extended survival we observed. Altogether, the therapeutic enhancement of signals 2 and 3 with the resulting effects on adoptively transferred tumor-specific T cells provides an explanation to the efficacy of this treatment to treat late-stage cancer in mice. Although the mechanism of action was not investigated in depth, the absence of survival benefit in TRAMP mice treated with anti-CD40 + IL-2cx + IL-12Fc without adoptive transfer strongly supports a CD8-dependent mechanism.

Although APC maturation, costimulation and cytokines are essential to avoid tolerance induction in T cells, the therapeutic approach described in this thesis may neglect an important cause of T cell tolerance. Indeed, naïve, but also differentiated effector T cells are tolerized in the context of cancer, most probably due to the plethora of immunosuppressive cells and molecules in the tumor microenvironment. This provides the rationale for further combining immunotherapies with conventional or targeted approaches to disrupt the tumor milieu and shift the balance towards a more immunosupportive setting. Local radiotherapy might be particularly well suited for this as it reduces the tumor load and at the same time promotes tumor-specific immunity (*61-63, 156, 157*). Other approaches to counteract the immunosuppressive signals of the tumor microenvironment include the targeting of immune-checkpoint proteins, such as PD-1, LAG-3 or adenosine 2A receptor to name a few (*83, 158*).

The recent approval of checkpoint blockade therapy with anti-CTLA-4 to treat metastatic melanomas represents an important milestone in the fight against cancer (*134, 149*). Subsequently, current clinical trials aim to broaden the indications for anti-CTLA-4 (*159-161*). However, as seen in melanoma patients, the frequency of responders to ipilimumab remains relatively low yet for unknown reasons, which suggests that other pathways must be targeted (*134, 149*). Following this line, PD-1 blockade, which as shown higher response rates including efficacy in ipilimumab-refractory patients and also better toxicity profile than anti-CTLA-4 (*136, 162, 163*), is now being combined with ipilimumab in humans (*84*). Based on these data and its approval by the Food and Drug Administration, anti-PD-1 (pembrolizumab) may represent a pillar for future combinations of immunotherapies.

A reason that may explain the relatively low success rate of ipilimumab lays in our incomplete understanding of its mechanism of action. For example, it was shown following its FDA-approval that anti-CTLA-4 efficacy relies not only on the blockade of its target, but also on the selective depletion of regulatory T cells due to their high expression of CTLA-4 (*125-127*). This mechanism probably explains why anti-CTLA-4 showed low efficacy in our experimental setup. Indeed, we used the anti-mouse CTLA-4 (clone UC10-4F10-11), which is an Armenian hamster IgG1 with weak affinity to murine FcγRs and therefore doesn't trigger ADCC. We also observed low efficacy with anti-PD-1 in TRAMP mice. However, in this case ADCC is not believed to be involved in the mechanism of action of this rat IgG2a antibody. Recent data from cancer patients being treated with blockade of the PD-1–PD-L1 axis indicate that PD-L1 expression in the tumor microenvironment correlates with better response rates (*45, 46, 144*). Based on this data, it is possible that the low efficacy of PD-1 blockade we observed in TRAMP mice is due to weak or absent PD-L1 expression in the tumor microenvironment (*164*).

The lack of biomarkers is another cause of low response rates with checkpoint blockade since it impairs the clinicians' ability to identify which patients benefit from this therapy. For example, recent data support the use of sequencing to establish the mutational load in melanoma patients for which anti-CTLA-4 treatment is being considered. In fact, higher mutational load is associated with a greater neoantigen landscape being presented by tumor cells and correlated with clinical benefit of anti-CTLA-4 immunotherapy (*47*). Subsequently, it is likely that the benefits of exome sequencing and identification of neoantigens will not be restricted to melanoma patients. Indeed, it is conceivable that a wider spectrum of neoantigens would correlate with clinical benefit in other types of cancer treated by immunomodulatory drugs.

Besides having the potential to be used to identify biomarkers, exome sequencing provides an opportunity to develop therapeutic cancer vaccines (*47, 165*). Many academic and industrial

groups are developing workflows to generate personalized vaccines for cancer patients. Following exome sequencing and identification of mutations, an algorithm is used to predict potential peptides that can bind MHC class I molecules. These predicted immunogenic peptides are then used to produce a vaccine in the goal of generating protective tumor-specific CD8 T cell immunity (*143*). Mass spectrometry analysis of the HLA ligandome of tumor cells can be used to validate the *in silico* predictions and represents an additional way to identify tumor-associated immunogenic peptides. Compared to sequencing and prediction algorithms, this method has the advantage of identifying non-mutant self-peptides as relevant tumor antigens (*166, 167*). Although this therapeutic vaccine approach represents a great opportunity to treat cancer on a personalized level, some limitations need to be addressed including prohibitive costs due to specialized equipment and labor-intensive methods, but also the predictive algorithms need to be improved to take into consideration the high allelic diversity of MHC molecules between individuals.

Both immunomodulation with checkpoint blockade and cancer vaccines ultimately aim at increasing the quantity and improving the function of tumor-specific T cells. Adoptive cell transfer represents another method to achieve this goal. As our study showed, adoptive transfer of naïve tumor-specific T cells can be efficient to eradicate late-stage cancer in mice when optimal signals are provided to these cells. However, the therapeutic rescue of tolerized $CD8^+$ T cells was more difficult to achieve than preventing tolerization. This suggests that *de novo* priming may be more efficient to mount protective tumor-specific immunity than rescuing pre-existing immunity. From a translational perspective, this represents a major issue since cancer is mainly diagnosed at stages when tumor-specific immunity is already deviated and providing a large amount of fresh tumor-specific cells remains challenging in a clinical setting. It is possible that lymphoablative conditioning of the host could improve responses to the combination of anti-CD40 + IL-2cx + IL-12Fc or to other immunotherapies by eliminating negative interference from various cells while inducing in parallel a strong influx of naïve T cells. The efficacy of preconditioning lymphodepletion in cancer therapy has been demonstrated in mice and humans receiving adoptive cell therapy using cyclophosphamide and fludarabine combined with total-body irradiation (*168, 169*).

Another category of adoptive cell transfer emerged by combining the current knowledge of T cell activation and methods of genetic engineering. Chimeric antigen receptors (CARs) were created to enhance the effector function of transduced T cells (CAR-T) while being directed towards a specific tumor-associated antigen (*170, 171*). CARs can target surface molecules via a single-chain variable fragment (scFv) derived from an antibody fused to a hinge, a transmembrane domain and intracellular costimulatory domains such as CD28, 4-1BB, CD3ζ which provides

signals 1 and 2 (*170*). The efficacy of this approach is well established, although not yet approved as a therapy (*172-175*). However, it suffers from the lack of known targets specifically expressed on the cell surface of tumor cells. Nonetheless, adoptive transfer of genetically engineered tumor-specific T cells has the potential to significantly improve clinical outcome for some cancers, but requires the identification of more tumor-specific targets.

Therapies based on adoptive transfers are remarkably effective in humans (*176-180*). However, the major risk with adoptive transfer of T cells consists of on-target, but off-tumor effects. This on-target/off-tumor toxicity is caused by T cell responses against normal tissue expressing the target antigen. It can cause severe immune-mediated adverse reactions (irAEs) and represents a limitation for the use of such therapy (*181-185*). A safer approach was developed recently by transducing an inducible suicide gene as a safety switch to avoid irAEs. The inducible Caspase 9 (iCasp9) system allows for the rapid and selective elimination of more than 90% of transduced T cells in less than 30 minutes with a single dose of the non-toxic synthetic drug, AP1903 (*186, 187*). This molecule triggers the dimerization of iCasp9 resulting in rapid induction of apoptosis in cells expressing the transgene. The safety and efficacy of this system was demonstrated in children who received genetically modified T cells to enhance immune reconstitution in the context of haploidentical stem-cell transplants as treatment for relapsing acute leukemia. The bioinert dimerizing drug was given to patients in whom graft-versus-host-disease (GVHD) developed following transplantation and showed that a single dose was sufficient to end GVHD and prevent recurrence (*187*).

Although the combination of adoptive T cell transfer with anti-CD40 + IL-2cx + IL-12Fc cured aggressive and poorly immunogenic late stage tumors in mice, its clinical translation remains difficult. Indeed, each compound of this combination can cause severe immune-related adverse events at high doses in humans (*188, 189*). This combination also causes side effects in mice that were not investigated thoroughly in our study, although we observed similar weight loss, splenomegaly and lymphocytosis than what was reported for anti-CD40 monotherapy (*190, 191*). The use of IL-2 complexes instead of recombinant IL-2 prevented pulmonary edema as described previously (*192*).

Most side effects associated with therapies including cytokines or checkpoint blockade involve T cells (*193-196*), are dose-dependent and cover a broad range in onset from sudden to multiple weeks after initiation of therapy (*188, 197*). Of note, there are conflicting data about irAEs significantly correlating with the probability of response to immunotherapy and prolonged overall survival (*197-201*). Depending on the grade and the organ(s) affected by irAEs, the strategies vary to limit damages, but corticosteroids like prednisone are the most prevalent approach. In the case of

cytokine-release syndrome, blocking of IL-6 receptor with a monoclonal antibody like Tocilizumab has been used effectively in patients with leukemia undergoing immunotherapy (*150*). Although these approaches can counteract treatment-related adverse events, toxicity still limits the number of patients benefiting from immunotherapy. Thus, how to achieve maximal clinical efficacy while causing manageable toxicity remains a major challenge in the field.

In conclusion, an optimal cancer immunotherapy should tackle multiple obstacles. 1) It should stimulate both the innate and adaptive arms of the immune system. 2) It should increase the frequency of fresh tumor-specific T cells and adoptive transfer or cancer vaccines may represent optimal ways of achieving this goal. 3) Therapeutic administration of compounds increasing the quality of costimulation is desirable. 4) Survival and differentiation of tumor-infiltrating T cells should be supported by local cytokine therapy. 5) Innovative delivery systems, careful dosage and safety switches may represent key enabling factors to limit immune-related adverse events and achieve maximal efficacy.

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9. Appendix

9.1. Dannenmann et al. 2013. Cancer Immunol Res. 1(5); 288–95

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Title: Spontaneous peripheral T-cell responses toward the tumor-associated antigen cyclin D1 in patients with clear cell renal cell carcinoma.

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Contribution: AB contributed to this manuscript by performing experiments to verify the presence of mutations in the gene coding for the tumor-associated antigen Cyclin D1 in multiple patients with clear cell renal cell carcinoma and to test the presence of cyclin D1–specific immunoglobulin G in patient sera by Western blotting. AB also contributed to write and review the manuscript.

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Cancer Immunology Research

Cancer Immunology Miniatures

Spontaneous Peripheral T-cell Responses toward the Tumor-Associated Antigen Cyclin D1 in Patients with Clear Cell Renal Cell Carcinoma

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Abstract

Renal cell carcinoma (RCC) is a heterogeneous group of kidney cancers with clear cell RCC (ccRCC) as the major subgroup. To expand the number of clinically relevant tumor-associated antigens (TAA) that can be targeted by immunotherapy, we analyzed samples from 23 patients with primary ccRCC for the expression and immunogenicity of various TAAs. We found high-frequency expression of *MAGE-A9* and *NY-ESO-1* in 36% and 55% of samples, respectively, and overexpression of *PRAME*, *RAGE-1*, *CA-IX*, *Cyclin D1*, *ADFP*, *C-MET*, and *RGS-5* in many of the tumor samples. We analyzed the blood of patients with HLA-A2$^+$ ccRCC for the presence of CD8$^+$ T cells specific for TAA-derived HLA-A2–restricted peptides and found spontaneous responses to cyclin D1 in 5 of 6 patients with *Cyclin D1*–positive tumors. Cyclin D1–specific CD8$^+$ T cells secreted TNF-α, IFN-γ, and interleukin-2 (IL-2), and degranulated, indicating the presence of polyfunctional tumor-specific CD8$^+$ T cells in the blood of these patients with ccRCC. The high frequency (43%) of *Cyclin D1* overexpression and the presence of functional cyclin D1–specific T cells in 83% of these patients with ccRCC suggest that cyclin D1 may be a target for immunotherapeutic strategies. *Cancer Immunol Res; 1(5); 288–95. ©2013 AACR.*

Introduction

The immune system recognizes and controls tumors through a process called cancer immune surveillance (1). Studies showed a correlation between tumor infiltration by T cells and patient survival in various cancers (2). Furthermore, spontaneous immunity against tumor-associated antigens (TAA) can be detected in patients with cancer (3). TAAs include cancer-testis (CT) antigens, differentiation antigens, mutated proteins, overexpressed proteins, and viral antigens (4). Boosting spontaneous TAA-specific immunity is a low-toxicity strategy that resulted in objective clinical responses in some patients with cancer (5).

Renal cell carcinoma (RCC) is a heterogeneous cancer that encompasses about 90% of all human kidney tumors, and clear cell RCC (ccRCC) is the major histologic subgroup (6). About one third of RCC has already metastasized at the time of diagnosis, and 20% to 50% of resected patients develop metastasis (7). Treatment of advanced and metastatic RCC is challenging due to its relative resistance to chemo- and radiotherapy (8).

Evidence supporting RCC as an immunogenic cancer includes cases of spontaneous regressions, increased incidence in immunosuppressed patients, and the high density of tumor-infiltrating leukocytes (TIL; ref. 9). Since the 1980s, immunostimulatory compounds such as interleukin-2 (IL-2) and IFN-α are being used to treat RCC. Despite modest therapeutic efficacy in some patients, the concomitant severe systemic toxicity remains a problem (8). Furthermore, data showing TAA-specific immunity in patients with RCC are scarce.

We conducted quantitative reverse transcription PCR (qRT-PCR) on samples from 23 patients with primary ccRCC for the expression of genes encoding cancer-testis antigens MAGE-A1 (CT1.1), -A3 (CT1.3), -A4 (CT1.4), -A9 (CT1.9), -A10 (CT1.10), synovial sarcoma X breakpoint 2 (SSX2/CT5.2), New York esophageal I (NY-ESO-1/CT6.1), L antigen 1 (LAGE-1/CT6.2) and MAGE-C1 (CT7), carbonic anhydrase IX (CA-IX/G250), renal antigen 1 (RAGE-1), preferentially expressed antigen of melanoma (PRAME), adipose differentiation-related protein (adipophilin/ADFP), C-MET proto-oncogene, cyclin D1 (CCND1), and regulator of G-protein signaling 5 (RGS-5). We also determined whether spontaneous T-cell responses were elicited against any of these antigens.

Materials and Methods

Patients

Patients with ccRCC underwent full or partial nephrectomy as part of their standard treatment at the Department of

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Note: Supplementary data for this article are available at Cancer Immunology Research Online (http://cancerimmunolres.aacrjournals.org/).

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Table 1. Patient information

Histology	ccRCC	
Patient number	$n = 23$	
Age, y	37–84 (64.1 ± 11)	
Pathologic stage	pT1	1
	pT1a	8
	pT1b	4
	pT2	3
	pT3	0
	pT3a	3
	pT3b	4
	pT3c	0
	pT4	0

Urology, University Hospital Zurich (Zurich, Switzerland), between 2008 and 2011. Tumor specimens and peripheral whole blood were obtained following informed consent in accordance with the Declaration of Helsinki. The local ethics committee approved the study (EK-1017 and EK-1634). Detailed patients' characteristics are listed in Table 1. All patients were HLA-A2–typed using fluorescein isothiocyanate (FITC)–conjugated HLA-A2–specific or isotype control antibodies (BioLegend), on a CyAn ADP 9 flow cytometer (Beckman Coulter), and data were analyzed using FlowJo software (TreeStar).

Processing of blood and tumor samples

Peripheral blood mononuclear cells (PBMC) were isolated from peripheral blood by Ficoll (Ficoll-Paque PLUS; GE Healthcare) density centrifugation. Immediately after surgery, a small piece of tumor tissue was snap-frozen in liquid nitrogen for RNA isolation. The remaining tumor was digested with 6 U/mL DNase I type IV (Sigma), 1 mg/mL collagenase IV (Sigma) in Dulbecco's modified Eagle medium (DMEM; Gibco), 50 U/mL penicillin, and 50 U/mL streptomycin (Gibco) for 1 to 2 hours at 37°C. The resulting single-cell suspensions and the PBMCs were cryopreserved at −80°C until analysis.

RNA isolation

Total RNA was extracted from snap-frozen ccRCC tumor samples using the RNeasy Mini Kit (Qiagen) followed by digestion with DNase I (New England BioLabs) according to the manufacturer's instructions.

Quantitative real-time PCR

The concentration and purity of RNA were evaluated using the NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies). Using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems), 500 ng of RNA was reverse transcribed according to the manufacturer's instructions; cDNA was stored at −20°C until qRT-PCR analysis was conducted on a Rotor-Gene Q real-time PCR cycler (Qiagen) using the commercially available TaqMan reagents with optimized primer and probe concentrations (TaqMan gene expression assays; Applied Biosystems; Table 2). After an initial hold for 2 minutes at 50°C and 10 minutes at 95°C, the probes were cycled 45 times at 95°C for 15 seconds and at 60°C for 60 seconds. All PCR reactions were carried out in triplicates. Threshold cycle (C_t) values were determined with the Rotor-Gene Q Series software 1.7. ΔC_t values were calculated by normalizing the

Table 2. TaqMan assays of TAAs and derived HLA-A2–restricted peptides

	TaqMan assay ID	Position	Peptide sequence	Peptide source
MAGE-A1	Hs00607097_m1	278–286	KVLEYVIKV	Not used
MAGE-A3	Hs00366532_m1	271–279	FLWGPRALV[d]	Not used
MAGE-A4	Hs00365979_m1	230–239	GVYDGREHTV	Not used
MAGE-A9	Hs00893224_m1	223–231	ALSVMGVYV	Thermo Scientific
MAGE-A10	Hs01560792_m1	254–262	GLYDGMEHL	Not used
SSX2	Hs00817683_m1	41–49	KASEKIFYV	Not used
NY-ESO-1	Hs00265824_m1	157–165	SLLMWITQC	Bio Synthesis
LAGE-1	Hs00535628_m1	41214	MLMAQEALAFL	Not used
		157–165	SLLMWITQV	Not used
MAGE-C1	Hs00193821_m1	959–968	ILFGISLREV	Not used
		1083–1091	KVVEFLAML	Not used
PRAME	Hs00196132_m1	425–433	SLLQHLIGL	Thermo Scientific
		100–108	VLDGLDVLL	Thermo Scientific
RAGE-1	Hs00179504_m1	32–40	PLPPARNGGL	Thermo Scientific
		352–360	LKLSGVVRL	Thermo Scientific
CA-IX	Hs00154208_m1	254–262	HLSTAFARV	Thermo Scientific
CCND1	Hs00765553_m1	101–109	LLGATCMFV	S. Stevanovic, Tuebingen
		228–236	RLTRFLSRV	S. Stevanovic, Tuebingen
ADFP	Hs00765634_m1	129–137	SVASTITGV	S. Stevanovic, Tuebingen
C-MET	Hs00179845_m1	654–662	YVDPVITSI	S. Stevanovic, Tuebingen
RGS-5	Hs00186212_m1	5–13	LAALPHSCL	S. Stevanovic, Tuebingen

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target mRNA levels to the endogenous control 18s-rRNA (Hs03928990_g1; Applied Biosystems). Testis cDNA generated from human testes total RNA (Invitrogen) served as positive control for expression analysis of cancer-testis antigens. As qRT-PCR for *MAGE-A9* suggested low expression in the healthy kidney control (CT 35, ΔCT 30), expression in ccRCC specimens was considered positive at a CT < 35 and a ΔC_t < 30. For overexpressed antigens, ΔC_t levels of tumors were compared with ΔC_t levels of healthy kidney control (human kidney total RNA; Invitrogen) using the $2^{-\Delta\Delta C_t}$ formula. Only changes 2-fold or more were considered as overexpression.

Genomic DNA extraction

Punch biopsies from paraffin-embedded ccRCC samples were incubated (10 minutes at 95°C) with 300 μL of buffer containing 20 mmol/L Tris pH 8.0, 20 mmol/L EDTA and 1% SDS. After cooling down, 3 μL of Proteinase K (18 ± 4 mg/mL; Roche) was added to each sample and incubated at 55°C for 72 hours. Digested samples were centrifuged and 4 volumes of RLT buffer (AllPrep DNA/RNA Mini Kit; Qiagen), were added to the supernatant. Genomic DNA was extracted following the manufacturer's protocol.

Cyclin D1 sequencing

Purified genomic DNA was amplified using the Phusion Hot Start High-Fidelity DNA Polymerase (Finnzymes) with the following PCR conditions: 10 minutes at 98°C, followed by 34 cycles of 30 seconds at 98°C, 30 seconds at 68°C, 20 seconds at 72°C with a final elongation of 5 minutes at 72°C. Sequences encoding the HLA-A2–restricted CTL epitope corresponding to cyclin D1 amino acids 101–109 or 228–236 were amplified by using the forward primer 5′-TGCGAGGAACAGAAGTGC-GA-3′/reverse primer 5′-TCCAGTGGTTACCAGCAGCTC-3′ or forward primer 5′-TGCTCACAGCCTCCTTCCCT-3′/reverse primer 5′-TCGGCATTTCCGTGGCACTA-3′ respectively. PCR products were purified using the QIAquick Gel Extraction Kit (Qiagen) and sequenced using the same amplification primers.

MACS sorting and *in vitro* stimulation

T-cell stimulation was conducted as described previously (10). Briefly, $CD8^+$ and $CD4^+$ T cells were isolated sequentially from PBMCs by positive selection using MACS (Miltenyi Biotech) according to the manufacturer's instructions. The remaining $CD8^-CD4^-$ fraction was used as antigen-presenting cells (APC), which were loaded with 10^{-5} mol/L of each peptide (Table 2). For *in vitro* stimulation, 5×10^5 $CD8^+$ T cells were incubated with 5×10^5 loaded and irradiated (30 Gy) APCs in 96-well flat-bottomed plates. Cells were cultured for 9 days in 200 μL of TC-RPMI [i.e., RPMI (Gibco), supplemented with $NaHCO_3$ (2 g/L; Sigma), L-glutamine (2 mmol/L, Sigma), penicillin and streptomycin (50 U/mL; Gibco), minimum essential medium nonessential amino acids (1×; Gibco), sodiumpyruvate (1 mmol/L; Gibco), 10^{-4} mol/L β-mercaptoethanol (Sigma), and 10% pooled human serum plus DNase I (6 U/mL; Sigma)]. T-APCs were generated from purified autologous $CD4^+$ T cells stimulated with 1 μg/mL phytohemagglutinin (PHA; Sigma) and 100 U/mL recombinant IL-2 (R&D Systems) during the 9-day culture. T-APCs were used as APCs during the short *in vitro* stimulation before intracellular cytokine staining (ICS). Medium was exchanged every second day with TC-RPMI containing 25 U/mL of IL-2.

Intracellular cytokine staining

At day 9, the presence of TAA-specific T cells was tested by short-term restimulation with relevant peptides, followed by ICS. Briefly, T cells were stimulated with peptide-loaded autologous T-APCs at a 1:2 ratio for 5 hours in the presence of brefeldin A (10 μg/mL; Sigma), monensin (10 μg/mL; Sigma), 1 μg/mL anti-CD28/49d (BD), and appropriately diluted phycoerythrin (PE)-labeled anti-CD107a antibody. Before addition to the assay, T-APCs were labeled with 2 μmol/L carboxyfluoresceinsuccinimidyl ester (CFSE; Sigma), which allowed their exclusion before analysis. Surface staining for CD45-PerCP, CD3-Pacific Orange (Invitrogen), CD8-ECD (Beckman Coulter), CD14-FITC, CD16-FITC, CD19-FITC, and live-dead staining (LIVE/DEAD Fixable Green Dead Cell Stain Kit; Invitrogen) was conducted in PBS [NaCl (136 mmol/L; Fluka), Na_2HPO_4 (8 mmol/L; Roth), KH_2PO_4 (1.5 mmol/L; Roth), pH 7] for 20 minutes at room temperature. Subsequently, cells were fixed with 4% formalin (Kantonsapotheke Zurich) and incubated for 5 minutes at room temperature with permeabilization buffer [PBS supplemented with 2 mmol/L EDTA (Sigma), 2% fetal calf serum (FCS; PAA Laboratories), 0.05% NaN_3 (Sigma), and 0.1% Saponin (Sigma)]. Intracellular staining was conducted using IFN-γ-APC, TNF-α-PECy7 (ebiosciences), and IL-2-Pacific Blue antibodies in permeabilization buffer for 20 minutes at room temperature in the dark. Cells were washed once with permeabilization buffer and resuspended in PBS containing 1% formalin. Samples were evaluated by flow cytometry and data were analyzed with FlowJo software as described earlier. Unless stated differently, all antibodies were purchased from BioLegend.

Tetramer staining

PBMCs or digested tumor samples were stained with tetramers for the presence of TAA-specific $CD8^+$ T cells. Briefly, cells were resuspended in PBS and incubated for 10 minutes at 37°C with PE-labeled tetramers consisting of HLA-A2 plus cyclin $D1_{101-109}$, cyclin $D1_{228-236}$, $CMV_{495-503}$ or $FLU\text{-}MATRIX_{58-66}$, followed by incubation with anti-CD45 APC, anti-CD8 Pacific Blue (both BioLegend), and live-dead stain (LIVE/DEAD Fixable Aqua Dead Cell Stain Kit; Invitrogen) for 15 minutes at 4°C. Cells were washed once with PBS and resuspended in PBS containing 1% formalin. Samples were evaluated by flow cytometry and data were analyzed with FlowJo software. The percentage of tetramer-positive cells was determined after gating on live, $CD45^+$ $CD8^+$ cells.

Western blotting

The presence of cyclin D1–specific immunoglobulin G (IgG) in patient sera was tested by Western blotting as described previously (11) using recombinant human cyclin D1 (Abnova cat. no. H00000595-PO1). Sera from 22 patients with ccRCC were used at a 1:250 dilution. MaxPab mouse polyclonal anti-

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human CCND1 (Abnova cat. no. H00000595-BO1P) was used as positive control at 1:1,000 dilution. Horseradish peroxidase (HRP)–conjugated goat anti-mouse IgG (Jackson ImmunoResearch; 115-035-008) was used as positive control and HRP-conjugated goat anti-human IgG (Jackson ImmunoResearch 109-035-006) for serum samples at a dilution of 1:25,000. Blots were developed with Western Lightning Plus enhanced chemiluminescence (ECL) reagents (PerkinElmer cat. no. NEL104001EA) and signals were measured using a Fusion FX7 machine (VILBER LOURMAT).

Results

Frequent expression of TAAs in primary ccRCC tumor samples

The expression of nine cancer-testis and seven other antigens in 23 primary ccRCC specimens was analyzed by qRT-PCR. We found expression of *MAGE-A9* and *NY-ESO-1* in 36% and 55% of samples, respectively, and did not find expression of the other seven cancer-testis antigens in any samples (Fig. 1A). Although *MAGE-A9* and *NY-ESO-1* were frequently expressed, their expression levels were very low (Fig. 1B). Overexpression of *RAGE-1* in 13%, *C-MET* in 30%, *PRAME* in 39%, *Cyclin D1* in 43%, *ADFP* in 65%, *RGS-5* in 83%, and *CA-IX* in 96% of ccRCCs specimens was also observed (Fig. 1A and B). More than 50% of the ccRCC specimens coexpressed four to six different TAAs (Fig. 1C). We found no evidence for preferred coexpression of particular TAAs (Fig. 1D).

Spontaneous cyclin D1–specific CD8$^+$ T-cell responses in patients with primary ccRCC

Because of limited specimen availability, we selected HLA-A2$^+$ patients and used previously described HLA-A2–restricted peptides for stimulation of PBMCs (http://www.cancerimmunity.org/CTdatabase; refs. 12–14; summarized in Table 2).

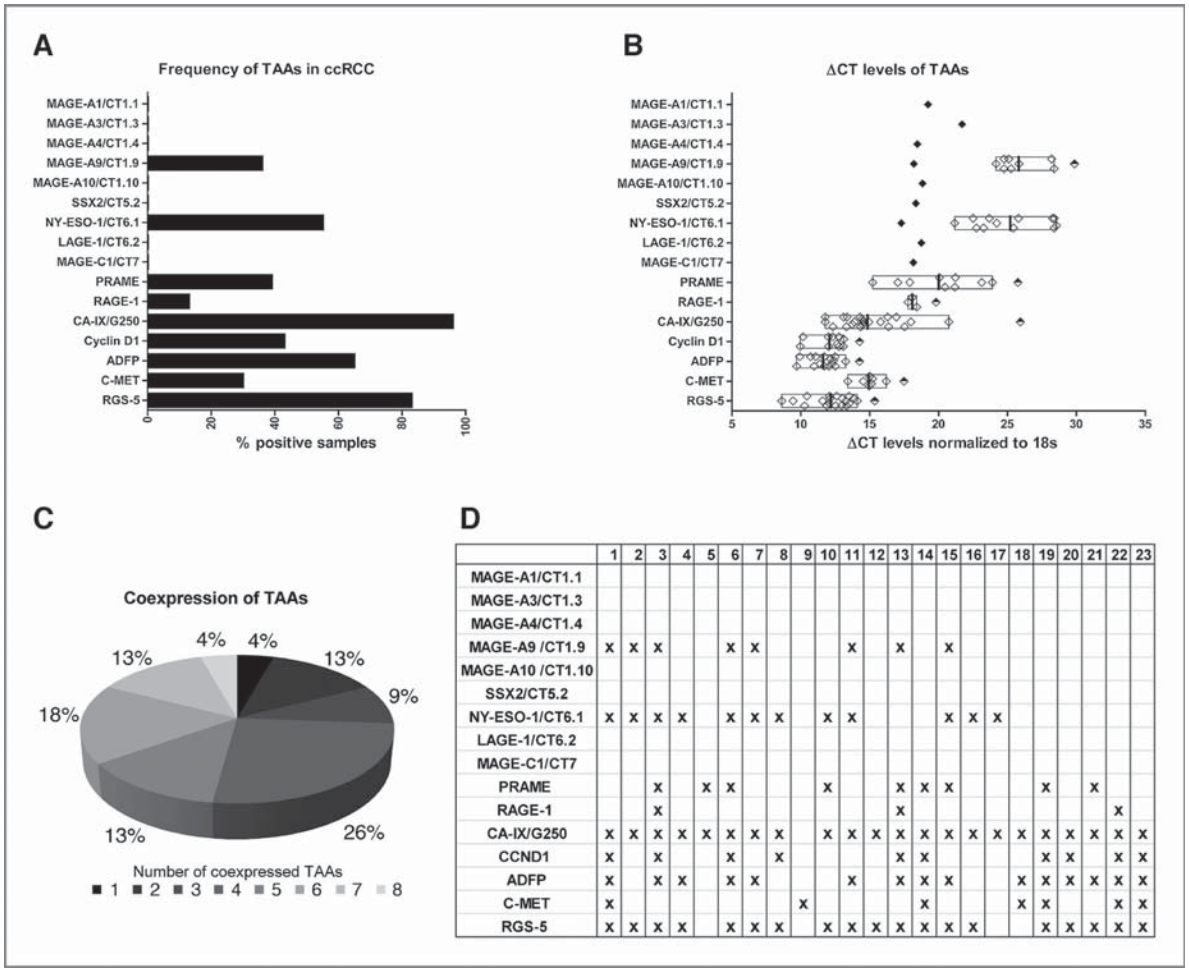

Figure 1. Expression of TAA transcripts in ccRCC specimens. RNA was isolated from 23 primary ccRCC samples and reverse transcribed, and cDNA was used as a template for qRT-PCR analysis. A, frequency of expressed TAAs in primary ccRCC samples. B, detected ΔCT levels for the different TAA transcripts of the individual tumor samples after normalization to the endogenous control (18SrRNA). Results from tumor samples are represented as open, from testis as filled, and from healthy kidney as half-filled symbols. C and D, coexpression of TAAs in primary ccRCC samples.

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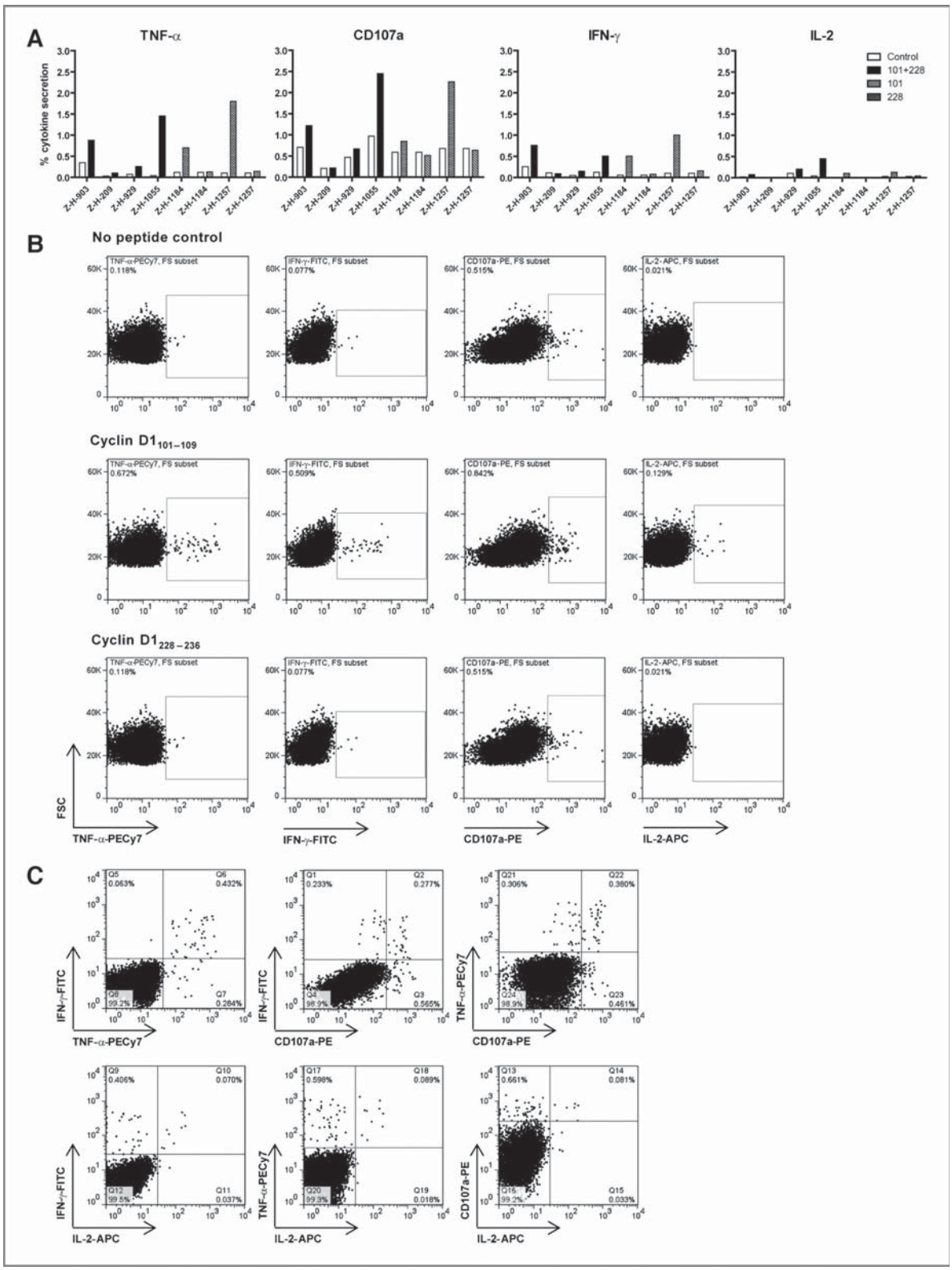

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After *in vitro* culture for 9 days with the relevant peptides, we conducted a 5-hour restimulation with the same peptides in the presence of brefeldin A and monensin, followed by ICS for effector cytokines TNF-α, IFN-γ, and IL-2, and staining for degranulation (surface CD107a). We found $CD8^+$ T cells specific for cyclin D1–derived peptides in 5 of 6 HLA-$A2^+$ patients, whose tumors overexpressed *Cyclin D1* (Fig. 2A), but not for any of the other peptides tested (data not shown). Because of limited material, the readout for patient samples Z-H-903, Z-H-209, Z-H-929, and Z-H-1055 was conducted with both cyclin D1–derived peptides (cyclin $D1_{101-109}$ and cyclin $D1_{228-236}$) together. Because there were enough specimens from patients Z-H-1184 and Z-H-1257, we analyzed the two cyclin D1–derived epitopes separately and found that responses in both patients were directed against cyclin $D1_{101-109}$ as illustrated by representative staining of specimens from patient Z-H-1184 (Fig. 2B). Furthermore, the cyclin D1–specific $CD8^+$ T cells in all 5 patients were polyfunctional although only a few cells secreted IL-2 (Fig. 2C). To exclude the possibility that the cyclin D1–specific $CD8^+$ T-cell responses were due to *in vitro* priming rather than *in vivo* induction by *Cyclin D1*–overexpressing tumors, we carried out a similar experiment with PBMCs from 3 healthy, HLA-$A2^+$ donors. $CMV_{495-503}$ was used as positive control because these donors were selected by the presence of CMV tetramer-specific $CD8^+$ T cells. We did not find cyclin D1–specific $CD8^+$ T-cell responses in these healthy donors, whereas CMV-specific $CD8^+$ responses were readily detectable by ICS (data not shown).

To confirm the presence of cyclin D1–specific T cells, we carried out *ex vivo* tetramer staining on PBMCs (Supplementary Fig. S1A) and TILs (Supplementary Fig. S1B) from patients with ccRCC when sufficient material was available. In PBMCs from patient Z-H-209, who did not show a cyclin D1–specific response after *in vitro* stimulation, we also did not detect cyclin D1 tetramer-positive T cells. However, in PBMCs from patients Z-H-903, Z-H-1055, and Z-H-1184, who responded to *in vitro* cyclin D1–specific peptide stimulation, we detected cyclin D1–specific T cells by tetramer staining (Supplementary Fig. S1A). Although we did not find cyclin D1 tetramer-specific T cells in TILs of patient Z-H-929, whose PBMCs responded weakly to cyclin D1–specific peptide stimulation *in vitro*, we detected cyclin D-1 tetramer-specific T cells in TILs of patients Z-H-1184 and Z-H-1257, whose PBMCs secreted cytokines upon peptide stimulation *in vitro* (Supplementary Fig. S1B).

To investigate whether cyclin D1 induced humoral immune response in patients with ccRCC, particularly those who overexpress *Cyclin D1*, we tested for cyclin D1–specific antibodies by Western blotting but did not find cyclin D1–specific antibodies in the sera of the 22 patients tested (Supplementary Fig. S2).

Discussion

To expand the number of clinically relevant TAAs that can be targeted by immunotherapy in patients with RCC, we investigated the expression of nine cancer-testis antigens and seven other antigens (15) in samples from 23 patients diagnosed with primary ccRCC. Most tumors expressed more than one TAA, which has been described for other tumor entities (16–18), and which enables therapeutic-targeting of multiple antigens at the same time.

Except for *MAGE-A9* and *NY-ESO-1*, we did not detect expression of the other seven cancer-testis antigens in our patient cohort. Although two studies showed expression for *MAGE-A3* and *-A4* in RCC (19, 20), our results are in line with those described in a previous review on cancer-testis antigens (21). The expression frequency of *MAGE-A9* in RCC is similar to that shown by Oehlrich and colleagues (12); however, we found only very low levels of *MAGE-A9* transcripts. As Oehlrich and colleagues have reported (12), we also detected a signal for *MAGE-A9* in the healthy kidney tissue control. The frequency of *NY-ESO-1* expression in CCR was unexpected on the basis of immunohistochemistry data published over a decade ago (22–24). The discrepancy may be explained by the highly sensitive qRT-PCR method we used that allowed the detection of very low levels of *NY-ESO-1* transcripts. Although *RAGE-1* was the first antigen recognized by autologous T cells from a human RCC cell line, it was only detected in 1 of 57 RCC samples (25). Our results (13%) are in agreement with studies that identified higher expression frequencies, which presumably is also due to the more sensitive methods used (12, 22). The frequency of *PRAME*-expression (39%) in ccRCC is in accordance with previously published reports (20, 22).

The von Hippel-Lindau (VHL) protein is mutated in most ccRCC samples, leading to reduced proteolytic degradation of hypoxia-inducible factor-α (HIF-α), resulting in upregulated HIF-α–mediated transcriptional programs (26). CA-IX is an HIF-α target gene that is frequently expressed in RCC but very rarely detected in normal kidney tissue (27). We observed an overexpression of *CA-IX* in all but one ccRCC samples. CA-IX has been identified as a therapeutic target for RCC (13, 28, 29), and evaluated in clinical studies (30–32), but it has not yet induced strong antitumor immune responses. In addition to *CA-IX*, *ADFP*, *Cyclin D1*, *C-MET*, and *RGS-5* are also overexpressed in ccRCC (14). In contrast to published reports (33, 34), we found that *ADFP* expressed more frequently (65%) than *C-MET* (30%). Cyclin D1 is a cell-cycle regulator crucial for the G_1–S transition (35) and is overexpressed in many cancers including colorectal and breast carcinoma (36, 37). We found that cyclin D1 was overexpressed in 43% of the primary ccRCC samples, a finding that was similar to the previous published data (38–40).

Many factors affect the success of immunotherapy (22). Despite the high frequency of expression (55%) of *NY-ESO-1*

Figure 2. Cyclin D1–specific $CD8^+$ T-cell response in PBMCs of patients with ccRCC. $CD8^+$ T cells from PBMCs of HLA-$A2^+$ patients with cyclin D1–overexpressing tumors were stimulated for 9 days with cyclin D1–derived peptides in the presence of IL-2. Restimulation was carried out using both cyclin D1–derived epitopes (cyclin $D1_{101-109}$ and cyclin $D1_{228-236}$) together, separately, or no peptide as highlighted in the figure, followed by surface staining for CD107a and intracellular staining for TNF-α, IFN-γ, and IL-2. Cytokine production of $CD8^+$ T cells was measured after gating on live $CD45^+$, $CD3^+$, $CD8^+$, $CD14^-$, $CD16^-$, and $CD19^-$ cells. A, summary of results from 6 individual patients. B, representative staining (patient Z-H-1184) of single cytokine secretion and (C) polyfunctional cytokine response.

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in ccRCC samples, which is one of the most immunogenic cancer-testis antigens (24, 41), we did not find NY-ESO-1–specific $CD8^+$ T-cell responses in any of the patients with HLA-$A2^+$ ccRCC, which may be explained by the low level of *NY-ESO-1* expression. ADFP, C-MET, and RSG-5 are not immunogenic in patients with ccRCC, as we did not detect T-cell responses specific for these antigens, despite their frequent overexpression. This finding is in agreement with previously published data on rare T-cell responses in patients with ccRCC (15), even though ADFP- and C-MET–specific T cells could be expanded from the blood of healthy donors (33, 34). *RGS-5* overexpression did not result in detectable RGS-5–specific T-cell responses in our cohort, although such responses were reported in the blood of healthy donors and patients with acute myeloid leukemia (42). In contrast, we detected cyclin D1–specific $CD8^+$ T cells in the blood of 5 of 6 patients with HLA-$A2^+$ ccRCC, whose tumors overexpressed *Cyclin D1*. Unlike cyclin-dependent kinase 4 (CDK4) in melanoma (43), we did not find a mutation in the two *Cyclin D1* epitopes in ccRCC (data not shown).

Ex vivo tetramer staining confirmed the presence of cyclin D1–specific T cells in patients whose $CD8^+$ PBMCs secreted cytokines upon *in vitro* cyclin D1–specific peptide stimulation. Although we detected a higher frequency of T cells specific for cyclin $D1_{228–236}$ by tetramer in blood and TILs, we found a higher frequency of T cells specific for Cycllin $D1_{101–109}$ after *in vitro* restimulation. Because cyclin $D1_{228–236}$ is the immunodominant peptide, it may induce more extensive *in vivo* proliferation of specific T cells and thus compromise their ability to further expand *in vitro*. Alternatively, tetramer-positive T cells may not be functional, suggesting a preferential functional exhaustion of T cells specific for cyclin $D1_{228–236}$.

There is no association identified between *Cyclin D1* expression and ccRCC prognosis (40), although *Cyclin D1* overexpression is associated with shorter patient survival in other cancers and thus represents an interesting therapeutic target (44). However, cyclin D1 is difficult to target as it is expressed in the cytosol and lacks intrinsic enzymatic activity. One approach is to block its activity indirectly by inhibiting associated kinases with kinase inhibitors (45); however, this strategy does not interfere with its kinase-independent tumor-promoting effects.

Cyclin D1 induces T-cell responses in mantle cell lymphoma and colon cancer, and antibodies in prostate cancer (46–49), and was identified as a target for immunotherapy in mantel cell lymphoma (50).

To our knowledge this is the first study that describes naturally occurring cyclin D1–specific $CD8^+$ T-cell responses in patients with cancer. Importantly, these responses have polyfunctional effector characteristics. We therefore propose cyclin D1 as a target for immunotherapy in patients with ccRCC.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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